Functional cross-talk between PTEN and PDK1 in normal haematopoiesis and leukaemia development

Lekgetho, Henry Kebafilwe

Awarding institution:
King's College London

Functional cross-talk between PTEN and PDK1 in normal haematopoiesis and leukaemia development

Henry Lekgetho

A thesis submitted for the Degree of Doctor of Philosophy

Department of Haematological Medicine
King's College London
2017

Declaration

I hereby declare that this thesis is my own work and has not been submitted to any institution for any award. Where other sources of information have been used, they have been acknowledged.

Henry Lekgetho

March/2017

Acknowledgments

Firstly, I would like to express my heartfelt gratitude to my supervisor Prof. Eric So for his support and motivation throughout my PhD. His guidance and patience helped me in the time of research and writing of this thesis. It was a great opportunity to be part of his research group.

I would also like to thank my second supervisor Dr. Yolanda Calle for her motivation and strength to carry on at hard time, my postgraduate coordinator Dr. Alan Ramsay for his encouragement and useful advices.

Above all, I would like to thank Dr. Bernd Zeisig for his guidance, patience and outstanding knowledge in research as well as his insightful comment during my thesis writing. My lab manager Miss Amanda Wilson for her hard work making sure I had everything I need and the extra hours she put in to make sure my work gets done. Nothing would have been possible without her.

Thank you to my previous and current lab mates who inspired and motivated me Dr. Clemence Virely, Mr. Winston Roy, Dr. Michael Cheung, Dr. Maria Teresa, Dr. Kar Lok Kong, Dr. Jackie Chen, Dr. Priscilla Lau, Dr. Magdalena Zarowiecki, Dr. Lu Zhao, Dr. Tsz Kan Fung, Dr. Jayant Rane, Dr. Maria Mallardo, Miss Clear Lynn, Dr. Abdullah Mobarki, Mr. Anthony Eiliazadeh, Dr. Teerapoong, Mr. Ray Tsai, Miss Si Yi Zhang, Mr. Nelson Ng, Dr. Marco Saia and Dr Chinh Hoang. Thanks guys for your help and I wish you all the best.

Last but not the least, I would like to thank my family: my parents and to my brothers and sister for supporting me spiritually throughout my PhD and my life in general. Special thanks to my daughter (Warona) and her mother (Edith) for their support and motivation to work harder and never to give up.

Abstract

PTEN is a tumour suppressor gene on chromosome 10, which has a central role in regulation of the phosphatidylinositol-3-kinase (PI3K) cell signalling pathway which has been linked to cell proliferation and apoptosis. Previous studies have shown that loss of PTEN induces leukaemogenesis by generating leukaemia stem cells and depleting haematopoietic stem cells. Phosphoinositide-dependent kinase-1(PDK1) is considered as a critical kinase in the PI3K signalling pathway and is responsible for mediating the phosphorylation of AKT and a wide range of other kinases that inhibit apoptosis. Given the important role of PI3K signalling in proliferation and apoptosis, we hypothesized that targeting of key components in this pathway such as PDK1 might be of therapeutic value in the treatment of loss of PTEN driven AML, ALL and other cancers. The main objective of this study is to investigate the role of PTEN and PDK1 in leukaemogenesis and normal haematopoiesis. We generated conditional knockout mice (PTEN $^{fl/fl}$ Rosa-cre-ERT), PDK1 (PDK1 $^{fl/fl}$ Rosa-cre-ERT) and PTEN-PDK1 (PTEN $^{fl/fl}$ PDK1 $^{fl/fl}$ Rosa-cre-ERT) where gene deletion can be mediated by tamoxifen treatment. To restrict the deletions to the haematopoietic compartment, bone marrow cells from the KO mice were transplanted to recipient mice and then treated with tamoxifen. Using these mouse models, we compared the impact of PTEN, PDK1 or both deletions on haematopoietic development from early HSCs to restricted progenitors. Further analysis using two specific knock-in (KI) mice (PDK1 L155e MG and PDK1 K465e) was carried out to further elucidate the activity of PDK1. In these models, PDK1 L155e MG mice have disrupted PDK1-PIF pocket domain whereas the PDK1 K465e have a defective PDK1-PH domain. These two domains are critical for the distinctive function of PDK1. Consistent with previously

published data, our data showed that loss of PTEN perturbed the normal haematopoietic development. Simultaneous deletion of PTEN and PDK1 rescued some of the abnormalities caused by PTEN deficiency suggesting that PDK1 may play a crucial role in haematopoiesis. We further demonstrate that loss of PTEN in the haematopoietic compartment results in the development of AML or ALL and in some cases both AML and ALL consistent with previous published data. In addition, we demonstrate that simultaneous deletion of PTEN and PDK1 does not completely reverse the leukaemogenesis induced by PTEN deletion; however it was able to significantly delay the onset of AML. Moreover, deletion of PDK1 in PTEN deficient haematopoietic cells rescued the mice from developing ALL in both $PTEN^{fl/fl}$ $PDK1^{fl/fl}$ Rosa-26cre-ERT mice and PTEN $^{fl/fl}$ PDK1 $^{fl/fl}$ Vav-cre mice. PDK1 MGK465 and PDK1L155 mutation did not have any effect on the phenotype of the leukaemia mediated by loss of PTEN, however both mutants significantly reduced the leukemic stem cell frequency of PTEN null ALL stem cells. Our data further suggest that the mechanism of leukaemia in the compound PTEN PDK1 KO might be due the ability to bypass the normal activation of Akt through PDK1 when PTEN is deleted. Further studies need to be carried out to identify the mechanism or cross talk that by passes PDK1 activation of downstream kinases when PTEN is deleted.

Table of Contents

List of Figures

List of Tables

Abbreviations

4EBP1	4E-binding protein-1
ABL	Abelson murine leukemia
AML	Acute myeloid leukaemia
APL	Acute promyelocytic leukemia
AKT/PKB	Protein Kinase B
BD	Binding Domain
BCR	Breakpoint cluster region
BM	Bone marrow
Bmi-1	B lymphoma Mo-MLV insertion region 1 homolog
CBF	Core binding factor
CBFB	Core Binding Factor Beta
CEBPα	CCAAT/enhancer binding protein alpha
CFU	Colony forming unit
CLP	Common lymphoid progenitors
CML	Chronic myeloid leukaemia
CREB	cAMP response element-binding protein
CMP	Common myeloid progenitors
Dll1	Delta like-1
Cre	Cre recombinase/Causes recombination
DEPTOR	DEP Domain Containing MTOR-Interacting Protein
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
ELDA	Extreme Limiting Dilution Analysis
ELF	eukaryotic translation initiating factor
ER	Estrogen receptor
ETO	Eight twenty one
ERK	extracellular signal–regulated kinases
FACS	Fluorescence-activated cell sorting
FAB	French-American-British
FLT3	FMS-related tyrosine kinase 3
FOXO	Forkhead box protein O
Gas7	Growth arrest-specific protein 7
CFC	Colony-Forming Cell
GM	Granulocyte macrophage
GM-CFC	Granulocyte-macrophage colony-stimulating factor
GMP	Granulocyte and myeloid progenitors
GSK3	Glycogen synthase kinase 3
HE	Hematoxylin and eosin
HOX	Homeobox
HR	Hit and Run
HSC	Haematopoietic stem cells
HSP90	Heat shock protein 90
IF	Immunofluorescence
IGF	insulin-like growth factor
IP	Immunoprecipitation
IR	insulin response

LDA	Limiting dilution assays
LMPP	Lymphoid-primed multi-potent progenitors
LSC	Leukaemia stem cells
LSK	Lin-Sca1+Kit+
LMPP	lymphoid-primed multipotent progenitor
LT-HSC	Long term-haematopoietic stem cell
MAPK	mitogen-activated protein kinase
MIC	morphologic, immunologic and cytogenetic
MEF	Mouse embryonic fibroblasts
MEP	Megakaryocyte/erythroid progenitor
MLL	Mixed lineage leukaemia
MPN	Myeloproliferative neoplasms
MPP	Multipotent progenitor
mTOR	mammalian target of rapamycin
MYC	Myelocytomatosis oncogene
NOD	Non-obese diabetic
NK	Natural killer cells
PBS	Phosphate buffer saline
PBD	Phosphate Binding domain
PcG	Polycomb group
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDK1	Phosphoinositide-dependent kinase-1
PH	pleckstrin homology
PI	Propidium iodine
PI3K	phosphatidylinositol-3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIF-pocket	Pocket in the kinase domain interacting with RSK,S6K,SGK and PKC
pIpC	polyinosinic:polycytidylic
PML	Promyelocytic leukaemia
PLK1	Polo like kinase -1
PRAS40	proline-rich AKT substrate of 40 KDa
PTEN	Phosphatase and tensin homolog
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
Rab	Ras-like GTPase proto-oncogene serine/threonine-protein kinase
Raf	Ras-like GTPase proto-oncogene serine/threonine-protein kinase
Raptor	regulatory-associated protein of mTOR
Rictor	Rapamycin-insensitive companion of mammalian target of rapamycin
RNA	Ribonucleic acid
RPMI	Roswell park memorial institute
RSK	Ribosomal s6 kinase-1 (p90)
RTK	Receptor tyrosine kinases
S6K	Ribosomal s6 kinase-1 (p70)
SCID	Severe combined immunodeficiency
SLAM	Signalling lymphocytic activation molecule
SM	Suspension medium
SP	Single positive
TAM	Tamoxifen
TCF	Transcription factor

TdT	Terminal deoxynucleotidyl transferase
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TSC	Tuberous sclerosis complex
WB	Western blot
WEHI	Walter and Elira Hall Institute
WHO	World Health Organisation
WT	Wild type
Wnt	Wingless type

Chapter 1 Introduction

1.1 Normal haematopoiesis and haematopoietic stem cells

Haematopoiesis is a process that generates and sustains all the blood cells of different lineages in the body. It is hierarchically organised with the haematopoietic stem cells (HSCs) at the apex possessing self-renewal properties and the ability to give rise to all progenitors and mature blood cells (Quesenberry et al., 2014). The self-renewal ability of HSCs may be achieved by undergoing an intrinsically asymmetric cell division whereby cell fate determinants are segregated into only one of the two daughter cells. While one daughter cell remains an HSC, the other daughter cell will proliferate to produce immature progenitors that gradually further differentiate into progenitor cells that are more lineage restricted and have lost self-renewal capability (Blank et al., 2008). However during developmental stage or injury additional self-renewal strategies that forcefully control the number of stem cells may permit them to undergo symmetric cell division to produce two daughter cells destined to the same fate, ie two HSCs or two differentiated cells (Morrison and Kimble, 2006) . The lineage restricted progenitors further proliferate and differentiate into mature functional blood cells (Yeung and So, 2009).

Several technological advances over the last few decades including the generation of monoclonal antibodies and fluorescent activated cell sorting (FACS) were instrumental to isolate phenotypically different haematopoietic cell populations. Functional characterization of the different cell types in the adult haematopoietic system (Fig 1.1) are based on results from in-vivo transplantation assays that demonstrated the ability of HSC's but not committed progenitors to fully reconstitute a lethally irradiated mouse. In addition, several in vitro differentiation assays were

made possible to further characterise haematopoietic cells by the discovery of haematopoietic growth factors (Robert-Moreno et al., 2008, Morgan et al., 2008). These studies demonstrated that Notch induction by adding soluble Jagged1 ligand to *ex vivo* cultures of human $CD34^{+}CD38^{-}Lin^{-}$ cord blood or incubating murine BM $Lin^{-}Sca1^{+}cKit^{+}$ (LSK) cells with a fusion protein containing the Dll1 ligand can expand haematopoietic progenitors as well as stem cells. In *vitro* expansion of these progenitor populations resulted in gain of function that allowed short-term hematopoietic reconstitution in mice following transplantation. The HSCs which possess unique combination of self-renewal and multi-lineage potential can be classified into long term or short term HSCs depending on their functionality. Long term HSCs (LT-HSCs) are characterised by their ability to provide long term multi-lineage reconstitution in a lethally irradiated mouse and reconstitute a secondary recipient mouse upon serial transplantation whereas short term HSCs (ST-HSCs) provide a short term reconstitution for many months after transplantation but cannot reconstitute a secondary recipient mouse (Yeung and So, 2009). All HSCs reside in the LSK (Lin^{-} $Sca1^{+}$ $c\text{-}Kit^{+}$) compartment in the murine (Fig 1.2) bone marrow and do not express mature cell surface markers such as CD19 and B220 found on B lymphoid cells, CD4, CD3 and CD8 found on the T lymphoid cells, Mac1 and CD15 expressed on the macrophages and Gr1 found on the granulocytes (Doulatov et al., 2012).

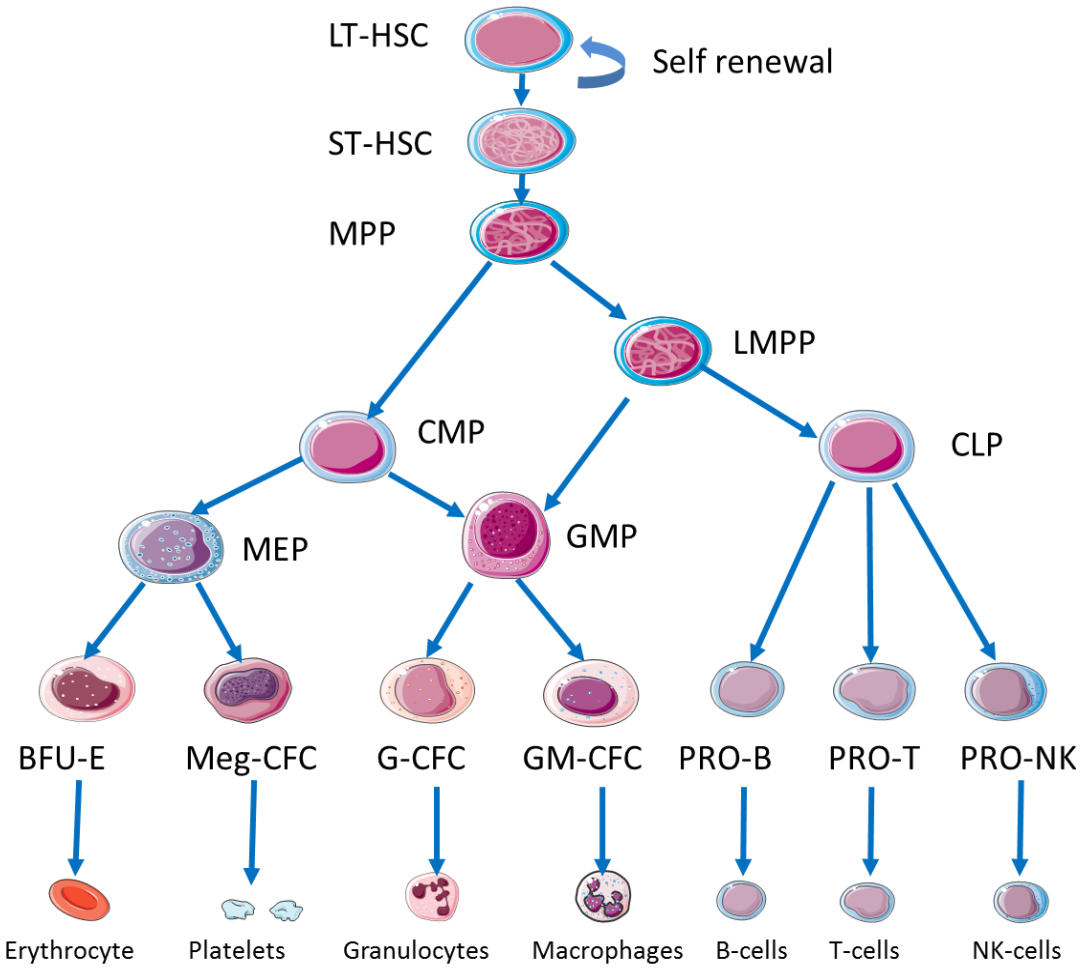


Figure 1.1: Schematic diagram of murine haematopoiesis

The HSC is located at the apex of the haematopoietic system and is the origin of all haematopoietic cells. Mature and functional blood cells are produced through a series of progressive proliferative and differentiation steps of the haematopoietic stem cells. All these proliferation and differentiation steps in normal haematopoiesis are tightly controlled and disturbances to this balance may result in haematological malignancies

Further functional analysis of the LSK population not only revealed the refinement of HSCs into LT-HSCs and ST-HSCs (see Table 1.1), but also identified non self-renewing multipotent progenitors (MPPs) downstream of HSCs (Fig 1.2). Signalling lymphocytic activation molecule (SLAM) family markers can be used to enhance the purification of mouse HSCs. SLAM family markers CD150 (also known as Slamf1) and CD48 (Slamf2) showed that LT-HSCs and ST-HSCs were enriched in the $CD150^{+}CD48^{-}$ and $CD150^{-}CD48^{-}$ fraction respectively while the MPPs are found in the $CD150^{-}CD48^{+}$ within the LSK compartment (Cai et al., 2011). More recently,

another population known as the LMPPs (lymphoid primed multipotent progenitors) has been found in the LSK compartment downstream of the MPPs (Yang et al., 2005, Doulatov et al., 2012). The LMPP population was identified as a more restricted progenitor within $CD150^{-}CD48^{+}$ population (Yilmaz et al., 2006a) that was positive for the surface marker FLT3 (Iwasaki and Akashi, 2007) (see table 1). LMPPs were able to give rise to cells of the myeloid and lymphoid lineages but had little or no potential to produce megakaryocytes and erythrocytes (Adolfsson et al., 2001).

Table 1.1 HSC and progenitor population in a normal adult mouse

Population	Cell surface marker
LT-HSC	Lin^{-} $Sca\text{-}1^{+}$ $c\text{-}kit^{+}$ $CD150^{+}CD48^{-}$
ST-HSC	Lin^{-} $Sca\text{-}1^{+}$ $c\text{-}kit^{+}$ $CD150^{-}$ $CD48^{-}$
MPP	Lin^{-} $Sca\text{-}1^{+}$ $c\text{-}kit^{+}$ $CD150^{-}$ $CD48^{+}$
LMPP	Lin^{-} $Sca\text{-}1^{+}$ $c\text{-}kit^{+}$ $CD150^{-}$ $CD48^{+}$ $(Flt\,3^{+})$
CLP	$Lin^{-/lo}$ $IL7R\alpha^{+}Sca1^{lo}c\text{-}Kit^{lo}$
CMP	$Lin^{-/lo}Sca\text{-}1^{lo}$ $c\text{-}kit^{+}$ $CD34^{+}$ $CD16/32^{lo}$
GMP	$Lin^{-/lo}Sca\text{-}1^{lo}$ $c\text{-}kit^{+}CD34^{+}$ $CD16/32^{hi}$
MEP	$Lin^{-/lo}Sca\text{-}1^{lo}$ $c\text{-}kit^{+}CD34^{-}$ $CD16/32^{lo}$

[a] Classification of HSCs and progenitors

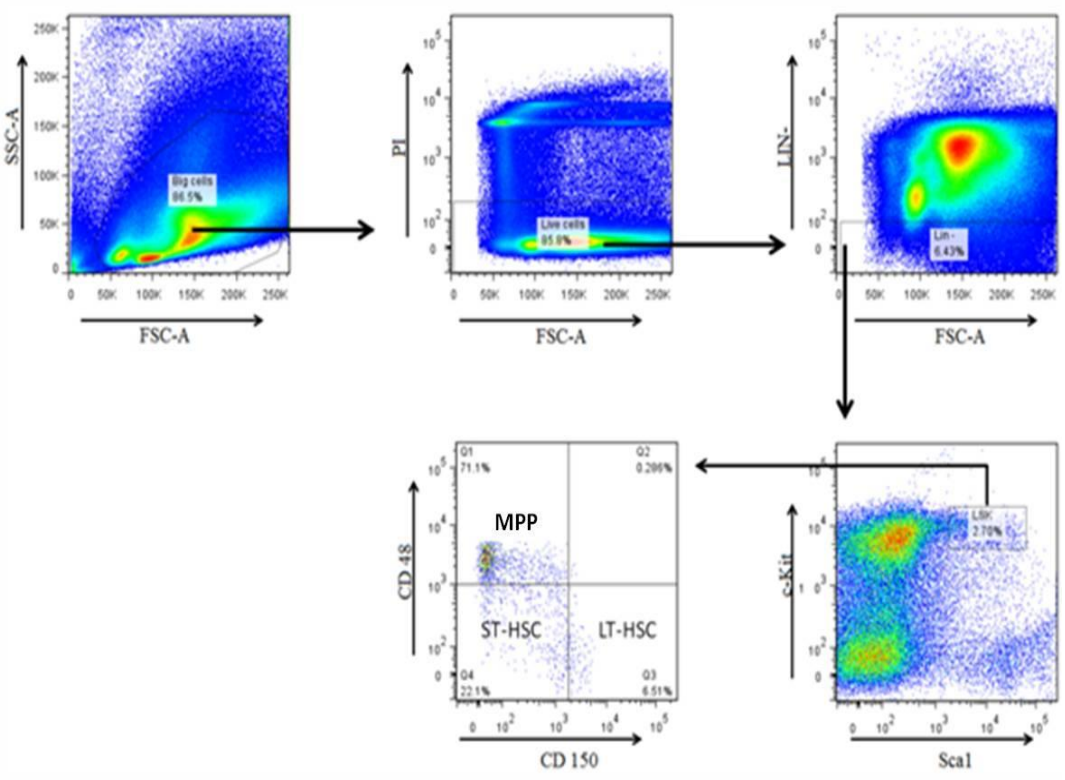


Figure 1.2: Classification of subpopulation of HSC

The HSCs are contained in the $Lin^{-/lo}$ $Sca1^{+}c\text{-}Kit^{+}$ (LSK) fraction of the bone marrow. Forward scatter (FSC-A) and side scatter (SSC-A) gates are set to remove debris. The PI gate is set to select live cells from dead cells. Sca1 and c-Kit positive cells are gated from the Lin^{-} cells fraction. LSKs are further classified into LT-HSCs and ST-HSCs MMPs and LMPPs by CD150 and CD48 expression. LT-HSCs to be enriched in the $CD150^{+}CD48^{-}$ and ST-HSCs enriched within $CD150^{-}CD48^{-}$ while the MPPs are found in the CD150-CD48+ within the LSK compartment.

The haematopoietic hierarchy branches to CMP (common myeloid progenitors) and CLP (common lymphoid progenitors) while CMPs give rise to megakaryocyte/erythrocyte progenitors (MEPs) or GMPs (Granulocyte macrophage progenitor); GMPs can also be derived from LMPPs. These progenitor populations are found in the lineage negative, Sca-1 negative and c-Kit positive fraction of the mouse bone marrow and make up to 20% of the total bone marrow cells (Yeung and So, 2009). Further classification of CMPs, GMPs and MEPs can be differentiated using CD34 and CD16/32 cell surface markers (Fig1.3). While CMPs are $CD34^{+}CD16/32^{lo}$, GMPs are $CD34^{+}CD16/32^{hi}$ and MEPs are $CD34^{-}CD16/32^{lo}$ (Table 1.1). On the other hand CLPs give raise to lymphoid restricted lineages known as pro-

B lymphoid cells and pro-T lymphoid cells. These will subsequently give rise to mature T cells, B cells and natural killer (NK) cells. CLPs constitute about 0.28 % of the total bone marrow cells and are enriched within the $Lin^{-/lo}$ IL-7Rα^{+}Sca-1^{lo} c-Kit ^{lo} compartment (Fig 1.4).

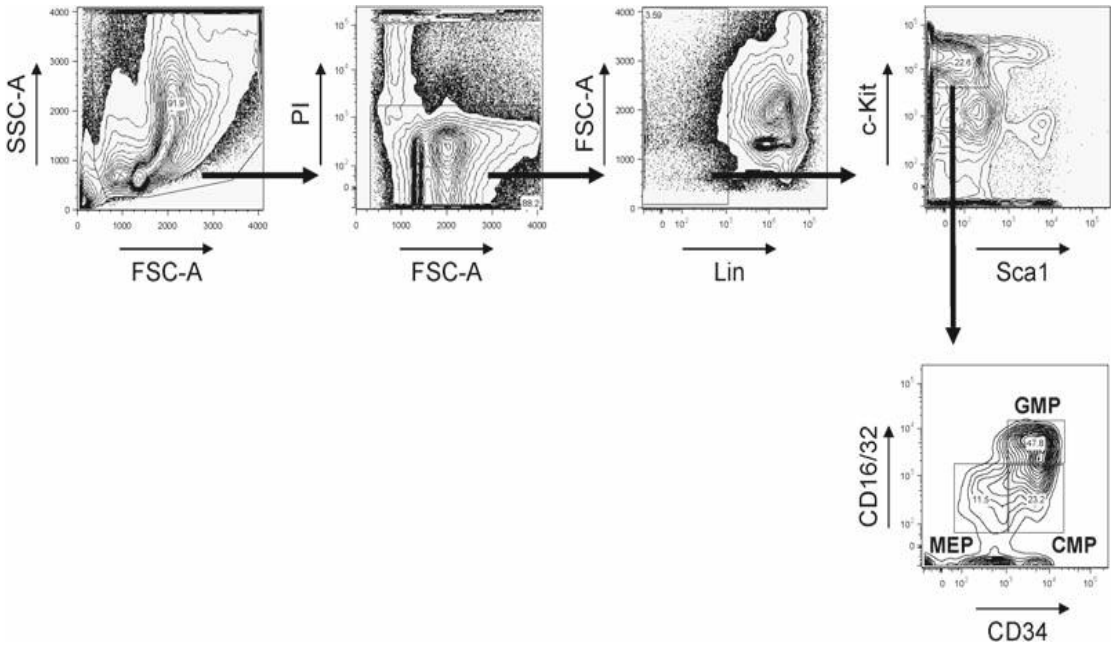


Figure 1.3: Classification of myeloid progenitor populations

The progenitor population is found in the lineage negative, Sca-1 negative and c-Kit positive fraction of the mouse bone marrow and make up to 20% of the total bone marrow cells (Yeung and So, 2009). Further classification of the progenitor population into CMPs, GMPs and MEPs can be made using CD34 and CD16/32 cell surface markers. While CMPs are $CD34^{+}$ $CD16/32^{lo}$, GMPs are $CD34^{+}$ $CD16/32^{hi}$ and MEPs are $CD34^{-}CD16/32^{lo}$.

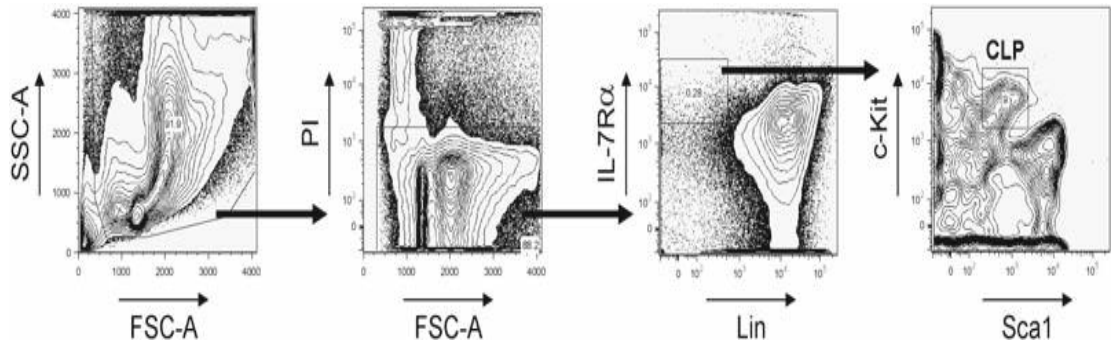


Figure 1.4. Classification of common lymphoid progenitors (CLP).

CLPs can be classified as $Lin^{-/lo}$ IL-7R$\alpha^{+}Sca^{lo}$c-Kit^{lo} cells and constitute 0.02% of the bone marrow (Yeung and So, 2009)

1.1.1 Pathways critical for haematopoiesis

Haematopoiesis is a tightly regulated process in which self-renewal, cell proliferation, differentiation and cell survival are maintained by several pathways and master regulators such as Bmi-1, Hox, p53 and p21, Wnt, Notch, Hedgehog, Raf/MEK, ERK and PI3 kinase pathways. Consistently, mutations in these pathways and master regulators may perturb the normal haematopoiesis process leading to various haematopoietic malignancies. Bmi-1 protein belongs to Polycomb Group (PcG) family and it is expressed mainly in the immature HSC population (Raaphorst, 2003, Park et al., 2003). Bmi-1 is necessary for the self-renewal of HSCs and it has been shown that Bmi1- knockout mice have defective haematopoiesis and develop hypocellular bone marrow (Park et al., 2003). In mammals, clustered homeobox (Hox) genes are organised in 4 clusters (HoxA to HoxD) and are tightly regulated in the haematopoietic system at each stage in lineage differentiation. Hox genes have been found to be frequently mutated and deregulated in haematopoietic malignancies (Eklund, 2011, Alharbi et al., 2013). Individual Hox genes seem to have diverse effect on haematopoiesis. For instance over expression of HoxA10 in mice results in

selective expansion of megakaryocytic cells with reduced monocytic and B-lymphoid progenitors (Borowitz et al., 1997), whereas over expression of hoxb4 results in the expansion of HSCs (Antonchuk et al., 2002).

The Wnt/β-catenin signalling pathway has been implicated in the regulation of haematopoiesis, however, different approaches by different laboratories yielded controversial and sometimes contradictory data, resulting in an incomplete picture of the actual role of Wnt/β-catenin in haematopoiesis (Staal and Luis, 2010). Wnt proteins fall under a large family of 19 soluble glycoproteins that have a wide range of effects on haematopoietic progenitors and haematopoietic stem cells. Although the Wnt/β-catenin pathway regulates self-renewal of various tissue stem cells and is active in HSCs (Perry et al., 2011), β-catenin itself is not required for self-renewal of adult HSCs (Cobas et al., 2004, Jeannet et al., 2008). Conversely, over-expression of β-catenin has also been associated with increased HSC frequency and prevention of differentiation (Reya et al., 2003), and was shown to predispose to T-cell lineage ALL (Guo et al., 2007). Moreover, WNT signalling is activated in AML cells by certain oncogenic fusion molecules such as PML-RARA or AML1-ETO (Kindler et al., 2005). A role for Notch signalling has also been proposed in the regulation of haematopoiesis and other tissues. While loss of Notch signalling pathway proteins Notch1 and Jagged-1 have been shown to result in defective HSC development at the embryonic stage in mice (Robert-Moreno et al., 2008, Kumano et al., 2003), over expression of intracellular Notch increased the number of HSCs, HSC self-renewal and favoured lymphoid over myeloid development (Stier et al., 2002). Moreover, Notch mutation has been suggested to be the main oncogenic lesion in T-ALL (Maillard et al., 2004).

The phosphotidyl-inosital-3 kinase (PI3K)/AKT pathway has also been linked with the regulation of haematopoiesis. The PI3K pathway is normally activated by the binding of ligands to receptor tyrosine kinases such as Fms-like tryrosine 3 (FLT3), c-Kit and is constitutively activated (without the need of ligand binding) by mutations of e.g. FLT3 such as FLT3-ITD. Consequently, FLT3 is one of the most frequent mutations in AML (Zeisig et al., 2012). Moreover, loss of PTEN, a negative regulator of the PI3K pathway leads to the generation of leukemic cells while depleting normal HSCs. It has also been speculated the PTEN might play a role in keeping the HSCs in a quiescent state (Zhang et al., 2006, Yilmaz et al., 2006b) as well as affecting the lineage selection. Furthermore, deletion of other components of the PI3K pathway such as AKT1/2, TSC1/2 as well as FOXa1, 2 and 3 in mouse HSCs has been shown to cause defects in HSC development (Warr et al., 2011).

1.2 Leukaemia and Leukemic stem cells

Leukaemia, generally classified as myeloid or lymphoid depending on the lineage involved and as acute or chronic based on the stage of differentiation of the malignant cells. Hematopoietic stem cells have self-renewal properties that allow them to persist throughout life. These self-renewal mechanisms are controlled by a system of master regulators that are often proto-oncogens and tumour suppressors (Zhang et al., 2009). Cancer cells hijack these mechanisms allowing them to transform into immortal cells that are able to self-renew just like HSCs. Interestingly, not all cancer cells within a cancer are equal. Data from several investigations has provided evidence suggesting that in any given cancer there is a variable fraction of phenotypically distinctive cell populations (e.g. AML 1/540 to Melanoma 1/3) that are more primitive compared with

the bulk of the tumour cells and exhibit properties similar to stem cells, i.e. self-renewal and differentiation (Grove and Vassiliou, 2014). Moreover, these rare and more primitive cells are often enriched for leukaemia initiating cells and are thus called leukemic stem cells (LSC).

In general, leukaemia is characterised by unrestrained proliferation of white blood cells in the bone marrow and these cells can be arrested at different stages of differentiation. Furthermore, LSC were first functionally identified and characterized in leukaemia by separating LSCs from the bulk of acute myeloid leukaemia (AML) cells and demonstrating that these cells resided only in the $CD34^{+}CD38^{-}$ population (Bonnet and Dick, 1997). While $CD34^{+}CD38^{-}$ fractionated patient cells were able to engraft into lethally irradiated non-obese diabetic-severe combined immunodeficient (NOD-SCID) mice and proliferate to repopulate the bone marrow of recipient mice with leukemic cells, mice injected with other CD34 CD38 populations failed to develop leukaemia (Dick, 2008). Although the exact LSC fraction may differ from patient to patient and may also depend on the mouse model used (Bonnet and Dick, 1997), these studies provide evidence of the existence of LSCs as the leukaemia initiating cells. The LSC model is clinically relevant as it can help to understand the high rates of both incomplete remission and more importantly relapses seen in the majority of leukaemia patients. While current chemotherapeutic agents target highly dividing cells both normal and cancerous, it has been shown that the LSCs may reside in the bone marrow microenvironment where they remain quiescent at G0 stage (Guan, Gerhard et al. 2003; Ishikawa, Yoshida et al. 2007) and thus evading the treatment and cause relapse after treatment(Sauer et al., 2015). Indeed, a better understanding of the LSC property is paramount for the development of targeted curative therapies

While LSC clearly share properties with normal HSC such as self-renewal and the ability to generate differentiated progeny, the cellular origin of LSC is unknown. Murine studies suggest that HSCs as well as myeloid restricted progenitors that have lost self-renewal capability can be transformed by chimeric proteins found in patients (So et al., 2003, Cozzio et al., 2003, Huntly et al., 2004, Sauer et al., 2015) to initiate leukaemia in lethally irradiated recipient mice. However, this is not a universal property, as not all oncogenic fusion proteins have the capacity to transform myeloid restricted progenitors to LSCs. For instance, BCR-ABL, the result of the t(9:22) translocation can only transform murine HSCs but not myeloid progenitors (Fig 1.5).

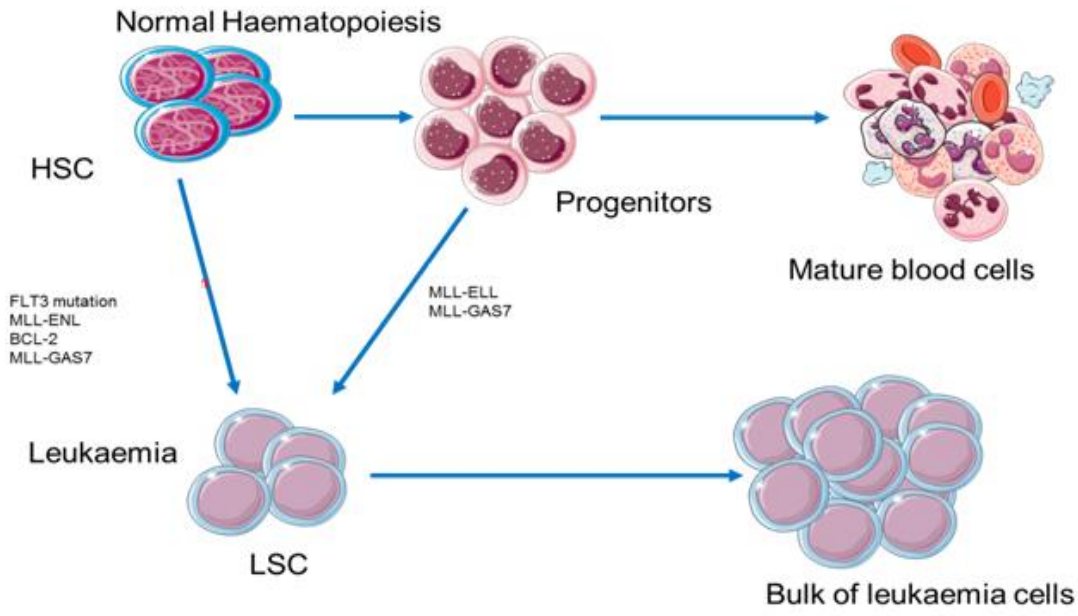


Figure 1.5: Schematic diagram of leukaemia stem cell origins.

Some oncogenic fusion proteins are able to transform normal HSC to LSC. Interestingly some other oncogenic fusion found in AML such as the MLL-Gas7 and MLL-ELL are transform HSC as well progenitors to LSCs. In general leukaemia can be viewed as haematological events initiated by a population of leukemic stem cells that have the ability like normal HSCs to self-renew and differentiate.

1.2.1 Acute Leukaemia

In contrast to chronic leukaemia that develops slowly over a number of years, acute leukaemia progresses rapidly and aggressively thus making early diagnosis and immediate treatment imperative. Acute leukaemia can be further classified into three subgroups: acute lymphocytic leukaemia (ALL), acute myeloid leukaemia (AML) and acute biphenotypic leukemia (ABL) depending on the expression of surface markers from the lymphoid and myeloid lineages.

1.2.2 Acute lymphoblastic leukaemia (ALL)

Acute lymphoblastic leukaemia (ALL) affects both children and adults, but 60% of cases occurs at ages < 20 with a peak at ages between 2 and 5 years (Inaba et al., 2013). ALL can be separated into two categories depending on the cells involved: T-ALL (T cell origin) or B-ALL (B cell origin). Advances in immunophenotyping, molecular cytogenetics and histology have contributed to a much more precise diagnosis and classification of ALL (see next section). More than 50 genetic alterations have been identified in ALL patients and gene deletions are among the most frequent aberrations (Mullighan et al., 2008). Several other mutations have been associated with ALL such as activating mutation in the PI3K pathway as well as the Notch1 signalling. In addition to mutations and deletions, ALL also results from chromosomal rearrangements that disrupt genes controlling normal haematopoiesis and lymphoid development such as MLL, AML1, MYC. Interestingly, these rearrangements result not only in the creation of fusion genes such as TEL-AML1 or

MLL fusions but also in translocations in which a gene (e.g. MYC or BCl-2) is brought under the control of immunoglobulin heavy locus or the T-cell antigen receptor gene locus in the case of TAL1, TLX1, TLX3 and LYL1 (Inaba et al., 2013).

1.2.3 Classification of ALL

The classification of ALL was first established on the bases of morphologic, immunologic and cytogenetic (MIC) presentation of the leukaemia (Szczepanski et al., 2003). It was later revised by the FAB (French, American and British) which made recommendation to classify ALL into sub-groups of ALL 1, 2 and 3. The inclusion of B- and T-lineage surface marker expression further refined the classification of ALL. While the morphological classification by FAB helped to distinguish different types of ALL, it has little clinical or prognostic relevance. Thus the WHO advocates, as an alternative, a classification that recognises clinically relevant molecular genetic characteristics of these lesions in addition to the morphology, immunology and cytogenetics.

Table 1.2 FAB and MIC classification of ALL

		Immunological marker						
Mic Group	FAB	CD2	CD7	CD10	CD19	TdT	clg	Karyotype
Early B- precursor ALL	L1, L2		-	+	+	+	-	t(4:11); t(9:22)
Common ALL	L1, L2		-	+	+	+	-	q- ; near-haploid; t(1:19);t(9:22)
PreB - ALL	L1		-	+	+	+	+	t(1:19); t(9:22)
B – cell ALL	L3		-	+/-	+	-	-	t(8:14); t(2:8) ; t(8:22)
Early T- precursor ALL	L1,L2	+	+		-	+		t/del(9p)
T – cell ALL	L1, L2	+	+		-	+		6q-

+, positive ; -, negative; no symbol, not specified by MIC
Abbreviations; TdT terminal deoxynucleotidyl transferase

Table1.3. World Health Organization (WHO) Classification of Acute lymphoblastic leukaemia with corresponding FAB classification

WHO classification	Corresponding FAB classification
Precursor lymphoblastic leukemia/ lymphoma	
Precursor B-cell acute lymphoblastic leukaemia/ lymphoma	L1, L2
Precursor T- cell acute lymphoblastic leukemia/ lymphoma	L1, L2
Burkitt's lymphoma/leukemia	
Endemic Burkitt's lymphoma/leukemia	L3
Sporadic lymphoma/leukemia	L3
Immunodeficiency- associated Burkitt's lymphoma/leukemia	L3

1.2.4 Acute Myeloid Leukaemia (AML)

AML occurs at any age accounting for approximately 20% of childhood leukaemia (Faulk et al., 2014) with increasing incidence in older adults. Both de novo as well as secondary AML (sAML) resulting from either the progression of other myeloid proliferative disorder such as myelodysplasia or the treatment of an unrelated neoplasm with topoisomerase II inhibitors has been described (Kumar, 2011). With the exception of t(15;17) PML-RARA subtype of AML for which overall survival rates of more than 5 years can be achieved for up to 80% of patients, the survival rates for all other subtypes is far worse. Chromosomal aberrations are found in about half of the AML patients and often involve transcription factors such as MLL, RARA and core binding factors (CBFs). In contrast to ALL, the recurrent translocations in AML result exclusively in the generation of chimeric fusion genes. While the other half of AML patients appears to possess a normal or complex karyotypes, next generation sequencing has identified aberrations in these patients that could not have been identified using karyotyping and banding, including mutations in signalling genes, transcription factors and epigenetic modifiers (Zeisig et al., 2012). Interestingly, receptor tyrosine kinases such as FLT3 and c-Kit upstream of the PI3 kinase pathway are one of the most frequent mutations in AML.

1.2.5 AML classification

Similar to the FAB classification of ALL, the FAB morphological classification in AML is only partially useful as it does provide common vocabulary and classification of the disease but has little clinical or prognostic relevance. Using the FAB classification, AML is confirmed when primitive blast cells are found in excess of more than 20% in the bone marrow. More recently, the WHO introduced a classification combining cytogenetics and molecular characteristics (Table 1.4). Chromosomal translocations leading to a gain or loss of function that affects normal haematopoiesis have been identified in over a third of AML patients (Look, 1997) and in most cases chromosomal translocation occur on genes that encode for transcription factors. In fact, the four most common translocations t(15;17) PML-RARA, t(8,21) AML1-ETO, inv(16) CBF-MYH11 and t(x;11) MLL fusions make up to 35% of all AML cases and have prognostic value. These and other mutations of prognostic relevance including mutations in the FLT3, NPM or CEBPA genes have been included in the WHO classification. Given that mutations in FLT3, C-Kit and PTEN, a negative regulator of the PI3K pathway, are found in over 30% of AML patients (Zeisig et al) and mutations of PTEN in over 20% of T-ALL (Palomero et al.). Deregulation of the PI3K pathway is a common event in AML and ALL. Indeed multiple PI3K inhibitors are currently being evaluated in clinical trials for various malignancies including haematological malignancies.

Table 1.4 French–American-British classification of acute myeloid leukaemia

Name	Description	Occurrence
M0	Acute myeloblastic leukemia, minimally differentiated	5%
M1	Acute myeloblastic leukemia without maturation	10%
M2	Acute myeloblastic leukemia with maturation	30% to 45%
M3	Acute promyelocytic leukemia（APL）	5% to 8%
M4	Acute myelomonocytic leukemia	15% to 25%
M5a and M5b	Acute monoblastic leukemia and acute monocytic leukemia	12% to 30%
M6 b	Acute erythroid leukemias	5% to 6%
M7	Acute megakaryoblastic leukemia	5%

Table 1.5 WHO classification

Name	Description
AML with recurrent cytogenetic translocations	• AML with t(8;21)(q22;q22)); RUNX1-RUNX1T1 • AML1/CBFalpha/ETO • Acute promyelocytic • AML with t(15;17)(q22;q12) and variants PML/RARalpha • AML with abnormal bone marrow eosinophils inv(16)(p13;q22) t(16;16)(p13;q22) CBFbeta/MYH1 • AML with 11q23 MLL abnormalities • Provisional entity: AML with mutated NPM1 • Provisional entity: AML with mutated CEBPA
AML with multilineage dysplasia	• With prior MDS or without prior MDS
AML with myelodysplastic syndrome, therapy related	• Alkylating agent related • Epipodophyllotoxin related • Other types
AML not otherwise categorized	• AML minimally differentiated • AML without maturation • AML with maturation (FT3 mutations) • Acute myelomonocytic leukemia • Acute monocytic leukemia • Acute erythroid leukemia • Acute megakaryocytic leukemia • Acute basophilic leukemia • Acute panmyelosis with myelofibrosis

http://www.cancer.gov/cancertopics/pdq/treatment/adultAML/healthprofessional/Page2#Section_119

1.3 Overview of the PI3K signalling pathway

The PTEN/PDK1/PI3K signalling pathway, controlling cell proliferation, growth and survival has been directly linked with cancer in several studies (Engelman, 2009, He et al., 2014, Hashimoto et al., 2014, Elkabets et al., 2013). PI3K pathway is often under normal circumstances activated by binding of ligands such as insulin, or platelet derived growth factor (PDGF) which will trigger the activation of the corresponding receptor tryrosin kinases (RTKs) (Fig 1.6). Likewise, other abundant receptors such as the insulin receptor (IR), FLT3 and c-Kit can also trigger the activation of the PI3K pathway. Genetic alterations in the upstream signalling molecules such as the RTK, FLT3,IR or c-Kit as well loss of PTEN have been associated with cancers including AML (McCubrey et al., 2012). The most critical PI3K proteins implicated in tumorigenesis and cell division are those that belong to class 1A which is formed by the catalytic subunit p110α and its associated regulating subunit p85 (Hafsi et al., 2012). Upon binding of a ligand to the tyrosine receptor kinases or associate receptor, a conformational change that allows the binding of p85 via its SH2 domain (Rous-sarcoma SRC oncogene homology 2 domain) will occur resulting in activation of the regulatory subunit. Once active p110α is released from its inhibitor (p85α), it is recruited to the plasma membrane (Vogt et al., 2006). Activated p110α phosphorylates Phosphotidylinisitil 4,5- biphosphate (PIP2) to Phosphatidylinisitol 3,4,5 triphosphate (PIP3) (Hales et al., 2013). PIP3 recruits adaptor and effector proteins phosphoinositide-dependant kinase 1 (PDK1) and protein kinase B (PKB/AKT) (McManus et al., 2004, Lawlor et al., 2002) . Interaction of PI3K with PDK1 results in a conformational change that allows PDK1 to phosphorylate AKT at Thr308 via the PH domain. To fully activate AKT, the mammalian target of rapamycin 2

(mTORC2) complex (Fig 1.6) is required to phosphorylate AKT at Ser473 (Hafsi et al., 2012) .

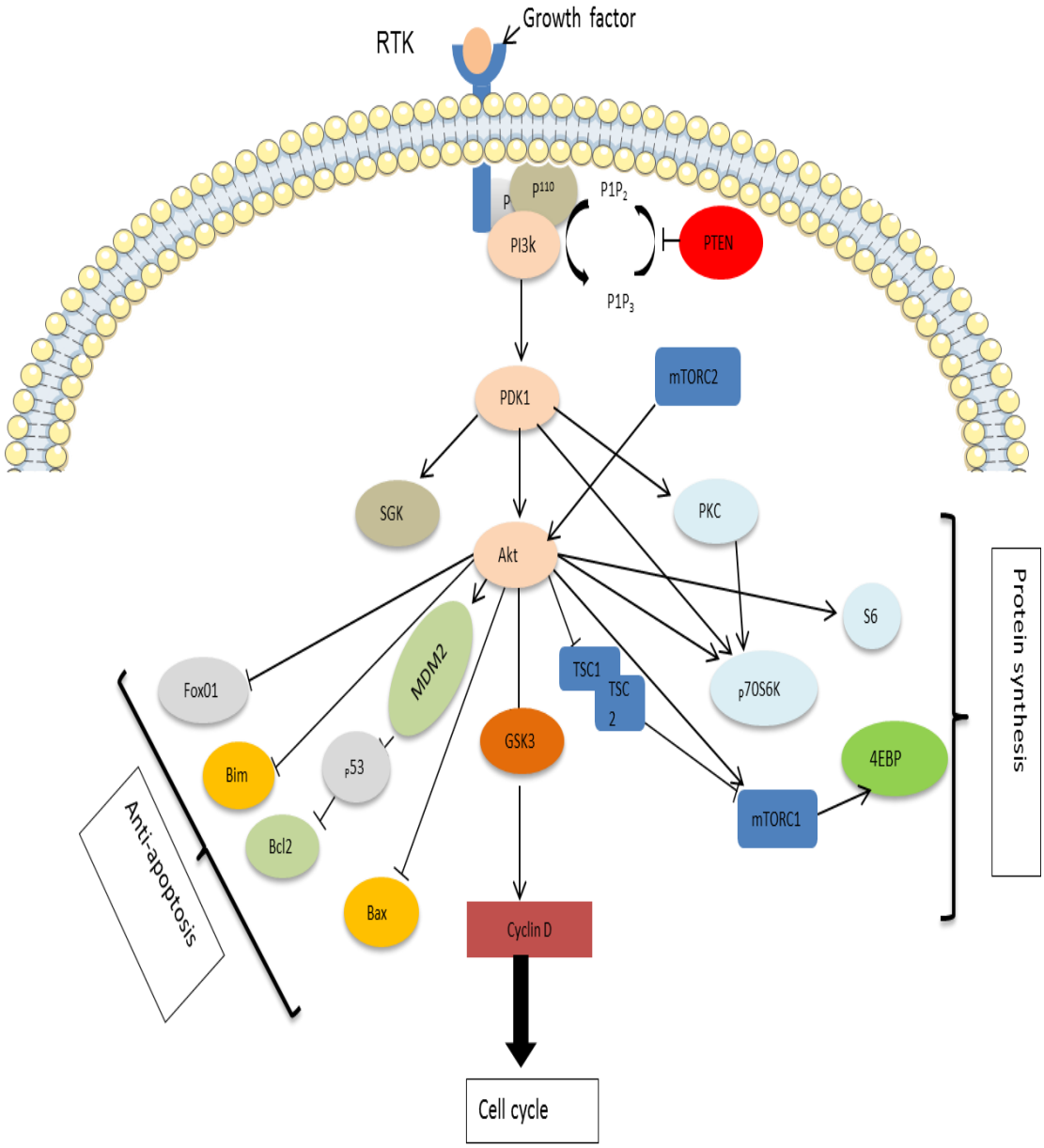


Figure 1.6: Schematic diagram of the PI3K pathway

PI3-kinase is often activated by binding of a ligand to the receptor tryrosin kinase (RTK).

In addition to AKT, PDK1 has been shown to phosphorylate a wide range of proteins known as AGC family kinases. These kinases include isoforms of protein kinase B, p70 ribosomal S6 kinase (S6K), p90 ribosomal S6 kinase RSK (Lawlor et al., 2002, Carracedo and Pandolfi, 2008). Activated AKT promotes cell cycle progression and cell proliferation through the phosphorylation of targets involved in protein synthesis, glycogen metabolism and cell cycle regulation (Kaul et al., 2012). Inhibition of GSK3 (glycogen synthase kinase 3) by AKT induces cell cycle progression by preventing the phosphorylation and degradation of β-catenin. GSK3 promotes the translocation of β-catenin into the nucleus where it forms a complex with different transcription factors such as TCF/LEF-1 to drive the expression of several genes that induce cell cycle progression such as Cyclin D1 and Myc (Perry et al., 2011). Inactive GSK3, phosphorylated by AKT, also increases glucose transporter Glut4 translocation to the plasma membrane by blocking AS160 (a160-kDa AKT substrate that has Rab-GTPase activity) (Karunanithi et al., 2014) and inhibiting phosphoenolpyruvate carboxykinase as well as glucose-6-phosphatase through inactivation of forkhead box O (FOXO) resulting in elevated glucose metabolism rate (Burgering, 2008). Most importantly, AKT activates mTOR which plays a critical role in regulating translation of proteins and ribosomal biogenesis (Kalaitzidis et al., 2012) by phosphorylating and blocking PRAS40 (proline-rich AKT substrate of 40 KDa) that inhibits the activity of mTOR. mTOR is a serine/threonine (Ser/Thr) protein kinase with important functions in haematopoiesis (Kalaitzidis et al., 2012).The mTOR kinase can be found in two multi-protein complexes named mTORC1 and mTORC2 (Fig 1.7). The mTORC1 complex is composed of 3 associated proteins, Raptor, PRAS40, and mLST8/GβL together with the mTOR catalytic subunit (Kalaitzidis et al., 2012, Harris and Lawrence, 2003). The mTORC2 complex however, contains mTOR and

mLST8/GβL, but instead of Raptor and PRAS40, it contains the proteins Rictor, mSin1, and Protor (Hung et al., 2012, Guertin and Sabatini, 2007). Both complexes are thought to be negatively regulated by DEPTOR (Wang et al., 2012). The mTORC2 is generally associated with the phosphorylation of Akt at S473 (Sarbassov et al., 2005). Conversely, Akt phosphorylates mTORC1 after its phosphorylation and activation by PDK1 at T308. An alternative activation of mTORC1 is through the inhibition of Tuberous sclerosis 1 (TSC1) and Tuberous sclerosis complex 2 (TSC2) (Fig 1.6) either by Akt itself or through the Ras/Erk pathway (Manning and Cantley, 2007) as well as activation by Rheb (Ras-homolog enrichedin brain) through the inhibition of FKBP38 which is also known to inhibit mTOR activity (Carracedo and Pandolfi, 2008).

Activated mTORC1 affects translation by phosphorylation of S6 kinase 1 and 2 as well as phosphorylation of 4E-binding protein 1 (4E-BP1) leading to the inhibition of eukaryotic translation initiating factor 4E (e1f4E) which has also been demonstrated to inhibit apoptotic (Glaysher et al., 2014). Hyper activation of mTORC1 in HSCs can evoke myeloid proliferative neoplasia (MPN) that may progress to AML. Mutations that lead to loss of function in the PTEN or TSC1 alleles (known inhibitors of mTORC1) or gain of function mutation in Akt and Rheb2 (known activators of mTORC1) in mouse HSC have resulted in HSC cycling and depletion of HSCs while generating leukemic stem cells (LSC) (Kalaitzidis et al., 2012). In mouse models where the function of mTOR1 complex was disrupted by deleting Raptor in the bone marrow, the mice developed a persistent and rapid pancytopenia, splenomegaly with extramedullary haematopoiesis and disrupted splenic architecture, and decreased thymic mass within 1 month (Kalaitzidis et al., 2012).

In addition, loss of Raptor led to increase frequency of both BM $CD48^{+}CD150^{+}$-, and $CD48^{+}CD150^{-}$ cells, while $CD48^{-}CD150^{+}FLT3^{-}CD34^{-}$-LT-HSC were largely unaffected. Interestingly mTORC1 loss via homozygous Raptor ablation is sufficient to extend survival in mouse models of PTEN loss evoked leukemogenesis in a cell-autonomous manner (Kalaitzidis et al., 2012). In mouse models where loss of mTOR2 was induced by ablation of Rictor all mice did not develop any signs of illness over the given period of time and showed no significant differences in bone marrow cellularity, HSC frequency, or absolute HSC numbers (Magee et al., 2012). While inhibitors of mTORC1 have been shown to be promising in cancer therapy (Guertin and Sabatini, 2009), it has also been demonstrated that substances such as rapamycin inhibiting mTORC1 have little or no effect on mTORC2, suggesting that mTORC1 and mTORC2 might be independently regulated.

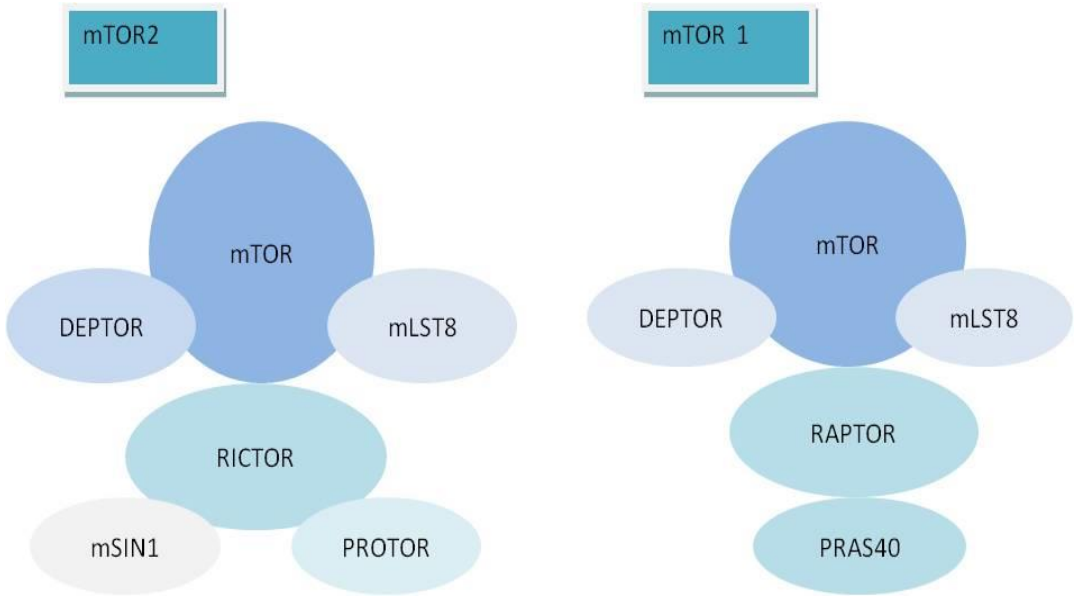


Figure 1.7: Schematic representation of mTOR 2 and mTOR 1 complexes

The mTORC1 complex is composed of 3 associated proteins, Raptor, PRAS40, and mLST8/GβL together with the mTOR catalytic subunit. The mTORC2 complex however, contains mTOR and mLST8/GβL, but instead of Raptor and PRAS40, it contains the proteins Rictor, mSin1, and Protor (Hung et al., 2012, Guertin and Sabatini, 2007).

1.3.1 Regulators of the PTEN/PDK1/PI3-Kinase signalling pathway

The prominent role of the PI3K pathway in cancer development strongly suggests that it is regulated by proteins with proto-oncogene or tumour suppressor functions. One of the most important tumour suppressors in this pathway is PTEN which is perhaps the main negative regulator of this signalling cascade. Loss of PTEN leads to uncontrolled PI3 kinase pathway signalling. PTEN itself is regulated at the transcriptional and post-translational level. Interestingly, part of its transcriptional regulation involves a negative feedback loop in which the transcription factors NF-κB and PPARβ/δ repress PTEN mRNA transcription and are themselves downstream of PI3 kinase signalling (Han et al., 2008, Barber et al., 2006). On the other hand, PTEN is also regulated at the post-translational level through ubiquitinlation- dependant degradation induced by the NEDD4-1, the only known ubiquitin E3 ligase for PTEN (Trotman et al., 2007). Moreover, NEDD4-1 mediated ubiquitination of PTEN allows it also to shuttle to the nucleus, whereas PI3K promotes the exclusion of PTEN from the nucleus (Shi et al., 2012), suggesting that PTEN activity is also regulated by its subcellular localization.

Another critical regulator of the PI3 kinase pathway is the tumour suppressor protein PML (promyelocytic leukaemia). PML was first identified as a component of PML-RARα fusion protein, the dominant initiating cytogenetic abnormality in acute promyelocytic leukaemia (Nakahara et al., 2014, Gamell et al., 2014). Recent studies have shown that PML is involved in various cell processes including regulating the DNA-damage response, apoptosis, senescence, and angiogenesis (Maarifi et al., 2014). In addition, PML has been reported as a regulator of metabolic pathways in stem cell compartments, including the hematopoietic system (Nakahara et al., 2014).

PML has been shown to affect apoptosis by recruiting PP2A phosphatase to the nucleus where it dephosphorylates and inactivates Akt residing in the nuclear bodies (Mazza and Pelicci, 2013). Inactivation and nuclear expulsion of Akt, has a considerable effect on functional properties of many tumour suppressor proteins such as FOXO3a. Absence of Akt allows for normal function of FOXO3a which is otherwise phosphorylated (at multiple serine and threonine residues) and deactivated by active nuclear Akt subjecting FOXO3a to degradation (Chatterjee et al., 2013). Repressed activity of FOXO3a results in the uncontrolled cellular proliferation coupled with reduced apoptosis. Deletion of PML gene by homologous recombination is not embryonic lethal and mice survive normally. However PML null HSCs have defective cell growth and differentiation (Nakahara et al., 2014). The PI3K pathway is also regulated through negative feedback from downstream effectors. The most well-known feedback regulating the PI3K pathway is triggered downstream of the mTORC1. Constant stimulation of the PI3K pathway has been shown to induce the phosphorylation of adaptor protein IRS 1 (insulin receptor substrate) leading to its proteasomal degradation. IRS is one of the adaptor proteins recruited to the RTKs during activation of the PI3K pathway. IRS1 transmits various key extracellular signals such as insulin and insulin-like growth factor (IGF) to downstream factors, subsequently activating intracellular signalling including the PI3 kinase pathway (Shen et al., 2014). IRS 1 is also regulated by S6K1 phophorylation, as phosphorylated IRS cannot bind to RTKs.

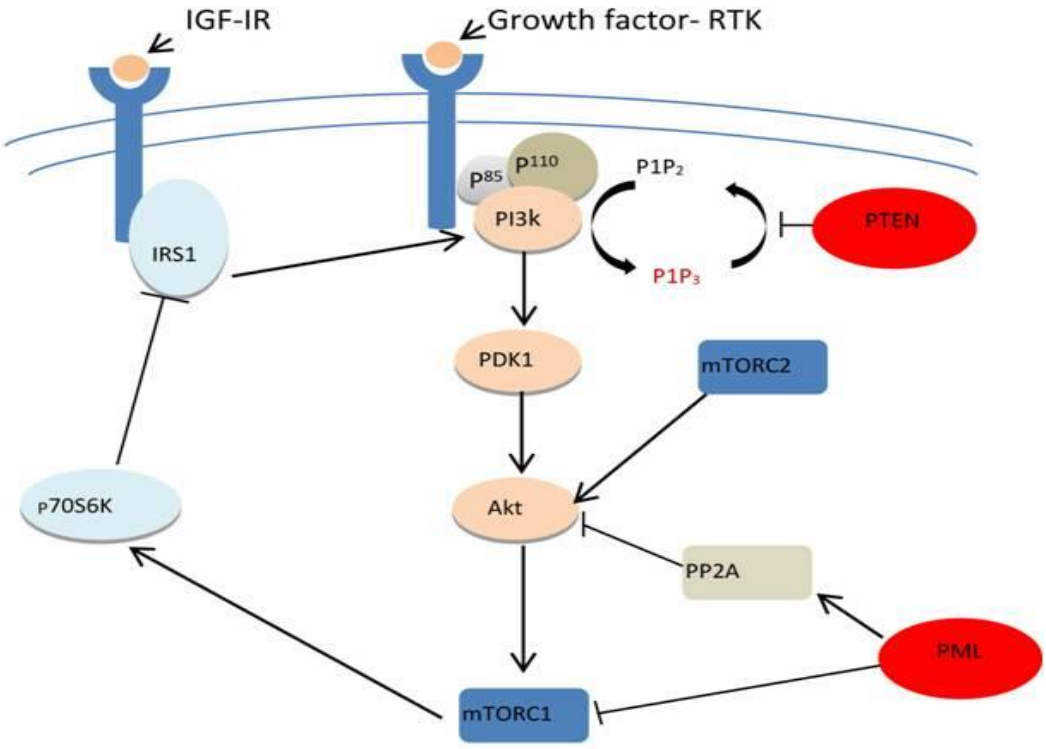


Figure 1.8: Feedback mechanism that counteract stimulation of PI3K.

An mTORC1/S6K-dependent negative feedback loop restraining IRS1-PI3K signalling.

1.3.2 Role of PTEN in tumorigenesis

While truncation, deletions or transcriptional silencing resulting in the loss of function of the tumour suppressor gene PTEN have been reported in several cancers (Dean et al., 2014, Ciuffreda et al., 2014, Cordes et al., 2013, Seront et al., 2013, Shuch et al., 2013, Chowdhury et al., 2013), specific mutations affecting the catalytic domain or ubiquitinlation site have also been associated with the development of malignant tumours (Trotman et al., 2007). Loss of PTEN leads to cell cycle check point failure therefore altering cell cycle kinetics as well as stimulating growth through the activation of the PI3K/Akt cascade (Gupta et al., 2009) . On the other hand over expression of PTEN can lead to reduced cell proliferation by arresting the cell cycle during the G_1 to S phase transition and also by inducing apoptosis due to reduced Akt

activity (Steelman et al., 2004) . PTEN mutations have been associated with various diseases including cancers. While PTEN mutation has been reported in more than 80% of patients with Cowden syndrome, a rare autosomal dominant inherited disease characterized by tumour-like growths and an increased risk of breast, thyroid, and endometrial cancer (Steelman et al., 2008), a pivotal role for PTEN has also been described in asthmatic bronchial inflammations (Kwak et al., 2003). PTEN is one of the most commonly lost tumour suppressors in human cancer with mutations rates of up to 50% in ovarian and endometrial cancers, up to 20% in endohyperplasia cancers (Steelman et al., 2004).

Recently, loss of function of PTEN has also been linked to leukaemogenesis. PTEN has been found to be mutated in human adult leukaemia but rarely in early childhood leukaemia (Magee et al., 2012). Deletion of PTEN from adult mouse hematopoietic cells has been shown to activate the PI3 kinase pathway leading to hematopoietic cell proliferation, depletion of HSC and leukaemogenesis (Zhang et al., 2006, Yilmaz et al., 2006b). Loss of function of PTEN has been frequently associated with T-ALL and albeit less often also with AML due to mutations or promoter methylation (Medyouf et al., 2010, Gutierrez et al., 2009), but is rarely seen in B-ALL which commonly presents in children. Given the contrasting role of PI3K pathway in various haematological malignancies and age of the patient, it has been speculated that the PI3K pathway may be differentially regulated during the course of life. Indeed, Magee and colleagues (Magee et al., 2012) demonstrated that PTEN is required for normal adult haematopoiesis but not for neonatal haematopoiesis using conditional knockout mice PTEN fl/fl Mx1–Cre in which PTEN was conditionally deleted after a single dose of pIpC at various time points after birth. PTEN deletion did not affect in vivo BrdU incorporation at 2 weeks after birth, PTEN- deficient HSCs showed a drift

towards increased BrdU incorporation at 3 weeks after birth which was further significantly enhanced at 4 and 5 weeks after birth. Furthermore, PTEN deletion induced in 2 week old mice resulted in a >40 fold increase in the number of mobilised HSCs in the spleen of mice compared to mice that were 6 weeks old when PTEN deletion was induced. On the other hand, when pIpC was administered to 2 days old neonate and 6 weeks old adult mice, histological signs of myeloproliferative disorder were observed in adult but not in neonatal spleen or liver 2 weeks after injection.

1.3.3 Role of PDK1 in tumogenesis

It has been shown that the interaction of PDK1 with its target proteins occurs at two main sites, the PH domain and the PIF domain (Fig 1.8). While the ability of PDK1 to phosphorylate S6K, SGK and RSK is dependent on the PIF-pocket (Biondi et al., 2001, Biondi et al., 2000, Collins et al., 2003, Najafov et al., 2012), phosphorylation of PKB and Akt is dependent on interaction with the PH domain (Bayascas et al., 2008, McManus et al., 2004). Activated Akt phosphorylates up to 100 substrates some of which inhibit apoptosis such as BAD, procaspase-9 and member of the fork head family of transcription factors (FOXO) (Brunet et al., 1999). Concurrently, Akt has been associated with increased resistance to apoptosis induced by TRAIL/APO-2L (TNF-Related Apoptosis-Inducing Ligand) (Xu et al., 2010). Furthermore, AKT also phosphorylates the transcription factors cyclic AMP response element-binding protein (CREB) and IκB (IKK) which regulate the expression of genes antagonising cell death (Caravatta et al., 2008). The importance of PDK1 in activating certain AGC kinase members was demonstrated by findings that mouse embryonic stem cells lacking

PDK1 failed to activate Akt, S6K and RSK whereas these enzymes were fully activated in wild type embryonic stem cells in response to stimulation with insulin like growth factor (Balendran et al., 2000). Due to the number and diversity of pathways regulated by PDK1, it has been proposed as a key oncogenic driver in some cancers (Ferro and Falasca, 2014, Tan et al., 2013). PDK1 has been found to be over expressed together with other effectors of PI3K pathway in PTEN deletion induced tumours (Chin et al., 2014, Tan et al., 2013). In addition, PDK1 has been shown to be essential for anchorage-independent and xenograft growth of breast cancer cells harbouring either PIK3CA or KRAS mutations (Gagliardi et al., 2012). Recently it has been reported that PDK1 directly phosphorylates the Polo-like kinase 1 (PLK1) which consequently leads to phosphorylation of MYC resulting in cell growth. MYC has been shown to be involved in pathways that control of self-renewal. Most importantly PDK1 directly activate Akt and mTOR complex 1, which have been directly linked to leukaemogenesis and depletion of normal haematopoietic stem cells (Yilmaz et al., 2006b).

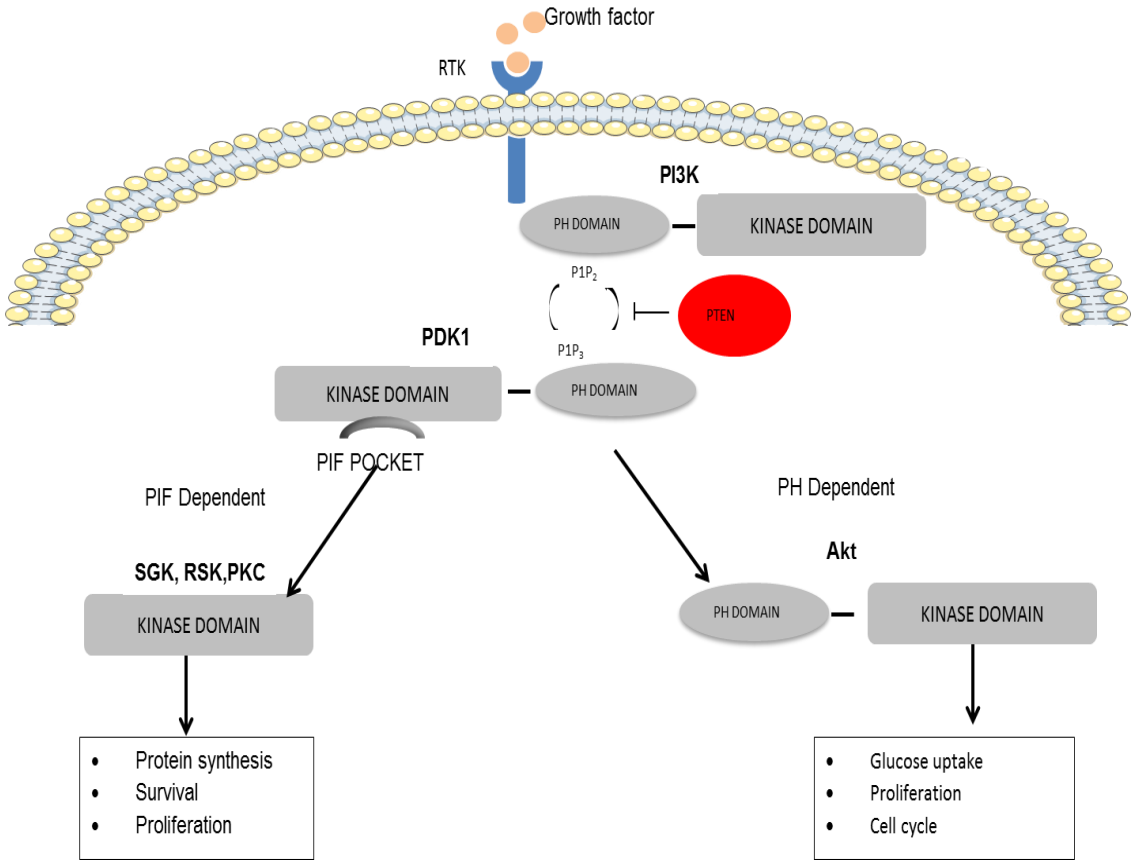


Figure 1.9: Structure and function of PDK1

The PIF pocket interacts with SGK, RSK and PKC whereas the PH domain interacts with Akt.

1.4 Targeting PTEN and PDK1 in cancer treatment

PTEN and PDK1 are major regulators of the PI3-kinase pathway and mutations or de-regulation of these proteins are frequently found in cancers. PDK1 contributes to cancer cell growth due to its ability to activate a series of signal transducers that are involved in promoting cell proliferation and survival. Because of its role, PDK1 has been identified as a promising cancer therapy target particularly in PTEN null cancers (Bayascas et al., 2005, Khan et al., 2013). Moreover, it has been demonstrated that conditional deletion of PDK1 rescues many effects of PTEN deletion in the brain (Chalhoub et al., 2009). In this study conditional deletion of PTEN alone in the brain

of mice resulted in macrocephaly, increased neuronal cell size and abnormal cerebellar neuron migration and layering, whereas PDK1 single knockout mice developed microcephaly (Iwanami et al., 2009). However, when both PTEN and PDK1 were knocked out, the mice had normal brain development with normal neuron cell size but still showed abnormal cerebellar migration, suggesting that PDK1 can rescue some but not all PTEN effects in normal murine brain development.

Using transgenic mice with the Lck-Cre system which allows PTEN deletion only in lymphocytes (Finlay et al., 2009) it was demonstrated that PTEN null mice die of lymphoma between 13 and 14 weeks. These mice had increased levels PDK1-Akt signalling indicated by elevated levels of threonine 308 and serine 473 phosphorylated Akt in comparison to the wild type mice. To address the importance of PDK1 in lymphoma caused by deletion of PTEN, mice that were genetically engineered to simultaneously delete floxed PTEN and PDK1 alleles in the T-cell progenitors were employed. While all PTEN null mice died of lymphoma, only a few mice with deletion of both PTEN and PDK1 developed lymphoma with a longer latency and the majority survived without any clinical symptoms of lymphoma, suggesting that PDK1 might be essential for the lymphomagenesis caused by PTEN deletion. Taken together, although these results show that loss of PDK1 does not entirely inhibit the whole spectrum of the aberrant pathology induced by PTEN loss of function, it can effectively prolong PTEN null lymphoma in mice, suggesting that PDK1 inhibition may be potent therapeutic strategy in PTEN null cancers. Given the involvement of PTEN in normal and malignant haematopoiesis, we propose to further dissect the role of PDK1 in PTEN deleted acute leukaemia and to expand the clinical utility of PDK1 inhibition.

1.4.1 PI3K pathway inhibitors

The PI3K-Akt pathway plays a vital role in cell proliferation and survival. Deregulation of this pathway has been reported in many cancers including colon, breast, lung and blood malignancies. Targeting this pathway has been shown to be of therapeutic value in the treatment of cancer. The first generation inhibitors of the PI3kinase LY294002 and wortmannin act as ATP inhibitors. Wortmannin binds to a conserved lysine residue in the ATP binding site of the enzyme PI3K therefore inhibits its activity (Wymann et al., 1996). LY294002 is a flavonoid derivative which acts as a ATP-competitive inhibitor that is also reversible (Wetzker and Rommel, 2004). LY294002 can effectively target other enzymes including the serine /therionine protein kinase (PIM1), protein-like kinase 1 (PLK1), casein kinase II (CK2) as well as ataxia-telangiectasia mutant (ATM) (Ogita and Lorusso, 2011). Both LY294002 and wortmannin have be shown to be highly effective PI3kinase inhibitors with anti-proliferative and apoptotic effect in vivo as well in vitro (Wetzker and Rommel, 2004). Moreover treatment of primary AML blasts with LY294002 has been show to induce apoptosis in vitro and also impaired engraftment in NOD/SCID mice (Xu et al., 2003). However due to the instability, poor solubility and toxic side effects the use of these two compounds has only been limited to preclinical application.

Recently other PI3K inhibitors are being studied by a number of academic institutions and pharmaceutical companies. Some of these inhibitors include pan-PI3K inhibitors targeting all four isoform of class 1 PI3K as well as isoform-selective inhibitors PI3Kα , PI3Kβ , PI3Kγ and PI3Kδ (Massacesi et al., 2016). Although all the four PI3K class 1A isoforms are known to be essential for the activation of AKT, p110δ has been

shown to be particularly consistently over expressed in AML. Interestingly, p110δ specific inhibitor IC87114 was able to repress the activation of AKT similarly to the effect of LY294002 and moreover impaired the proliferation of AML blasts (Billottet et al., 2006, Sujobert et al., 2005). Inhibitors targeting AKT directly have also been developed and some are already in pre-clinical or early phase clinical trials. AKT inhibitors are classified as either alkylysophospholipis (APLs) or small molecule inhibitors. (Barrett et al., 2012). APLs mainly act on the cellular membrane of actively dividing cells by blocking the recruitment of AKT to the cell membrane where it gets activated. A few AKT small molecule inhibitors have been used in clinical trials including GSK2141795, MK-2206 and triciribine (Barrett et al., 2012). Other small molecular inhibitors targeting PDK1 which is responsible for the activation of AKT have also been developed including GSK2334470 and AR12.

Objectives

Tissue homeostasis and regeneration are tightly controlled processes and their disruption contributes to the development of malignancies. Several pathways and key molecules such as Bmi-1, Hox, Wnt, Notch, Hedgehog, and the PI3 kinase contribute to the regulation of haematopoiesis. The PI3 kinase pathway is frequently found up-regulated in cancers including acute leukaemia, where mutations in upstream RTKs and loss of PTEN have been identified as the main causes for aberrant activation of the pathway. Given the crucial role of PDK1 in mediating PTEN functions, we hypothesize that PDK1 which relay upstream signals to downstream kinases and effector proteins might be a therapeutic target in the treatment of AML and other cancers which display activated PI3K pathway activity. Thus we will investigate the consequences of loss of PDK1 in the development of PTEN loss mediated leukaemia using various genetic mouse models. The main objective will be to define the contribution and critical pathways mediated by PDK1 in the establishment of LSCs induced by loss of PTEN.

The aims of my PhD project are

- To investigate the role of PTEN and PDK1 in leukaemogenesis and normal haematopoiesis
- To explore the origin of the leukaemia stem cells generated by loss of PTEN.
- To identify other potential kinases that might play a role in PTEN deletion mediated leukaemia

Chapter 2 Materials and Methods

2.1 Rosa-26cre-ERT mice

The Rosa-26cre-ERT mice carry a cre recombinase (Cre-ERT2) allele inserted into the universally expressed Rosa26 locus. Cre is a 38kDa enzyme that belongs to the integrase family of site specific recombinase that catalysis the site specific recombination event between two DNA recognition sites (loxP sites). The estrogen receptor T2 moiety fused to cre (CRE-ERT2) is retained in the cytosol and suppressed by binding with the heat shock protein 90 (hsp90) until tamoxifen administration releases its inhibition, thus permitting inducible recombination of loxP sites(Meinke et al., 2016). In this study we crossed the Rosa-26cre-ERT mouse with a C57/BL6 mouse carrying a gene of interest flanked by two loxP sites to generate conditional knockout mice. The following primers (forward 5'-AGATGCCAGGACATCAGGAACCTG-3' Reverse 5'-ATCAGCCACACCA GACACAGAGATC3') binding the *Cre* coding regions were used on genomic DNA isolated from biopsies to detect the Cre-ERT allele, producing a 400bp PCR product. The following PCR programme was used: denaturing at 94ºC for 5 minutes, 35 cycles of 94ºC for 30 seconds, annealing at 54ºC for 1 minute, extension at 72ºC for 2 minutes, final annealing at 72ºC for 5 minutes and hold at 4ºC.

2.2 Vav-cre mice

Vav-cre-mediated recombination occurs in most hematopoietic cells in all haematopoietic organs, including the haematopoietic progenitor-rich bone marrow. Vav-cre is a non-inducible cre system that is active mainly in the haematopoietic cells with limited activity in the vascular endothelium but not in other tissues (Georgiades et al., 2002). We crossed a Vav-cre mouse with a C57/BL6 mouse carrying a gene of interest flanked by two loxP sites to generate transgenic mice that lose the expression of the gene of interest predominantly in the haematopoietic cells. The following primers (forward 5'-AGATGCCAGGACATCAGGAAC 3' Reverse 5'ATCAGCCACACCAGACAC AGA - 3') binding the cre coding regions were used on genomic DNA isolated from biopsies to detect the Cre allele, producing a 210 bp PCR product. The following PCR programme was used: denaturing at 94°C for 10 minutes, 35 cycles of 94ºC for 40 seconds, annealing at 62ºC for 40 seconds, extension at 72ºC for 50 seconds, final annealing at 72ºC for 5 minutes and hold at 4ºC.

2.3 $PTEN^{fl/fl}$ Rosa-26cre-ERT and $PTEN^{fl/fl}$ Vav-cre

We generated conditional PTEN knockout mice using the Rosa-26cre-ERT system as well as the Vav-cre system. In the $PTEN^{fl/fl}$ mice, exon 5(Fig 2.1A) of the PTEN allele encoding the phosphatase domain (Fig 2.1B) is flanked by *loxP* sites. $PTEN^{fl/fl}$ females were crossed with males that carry a single Rosa-26cre-ERT or Vav-cre recombinase to generate first generation progeny $PTEN^{fl/wt}$ Cre+. $PTEN^{fl/fl}$ Cre+ progeny was achievable on second generation and subsequent generation by mating $PTEN^{fl/fl}$ with

PTEN$^{fl/wt}$ Cre+. Both mouse lines were born without any physical deformation. For the analysis of the deletion of PTEN gene at the DNA level, genomic DNA was extracted from blood and ear biopsy and amplified by PCR. Forward primer (5'-GTGAAAGTGCCCCAACATAAGG-3') and reverse primer (5'-CTCCCACCAATG AACAAAVAGT-3') were used to detect the wild type (WT) and floxed PTEN alleles producing a 428 bp product for the wild type and 514 bp product for the floxed allele. For detection of the deleted allele, forward primer (5′-GGCCTAGGACTCACTAGATAGC-3′) and reverse primer (5′-CTCCCACCAATGAACAAAVAGT-3′) were used producing a 705 bp product. The following PCR programme was used: denaturing at 94ºC for 4 minutes, 35 cycles of 94ºC for 30 seconds annealing at 60ºC for 30 seconds, extension at 72ºC 30 seconds, final annealing at 72ºC for 5 minutes and hold at 4ºC.

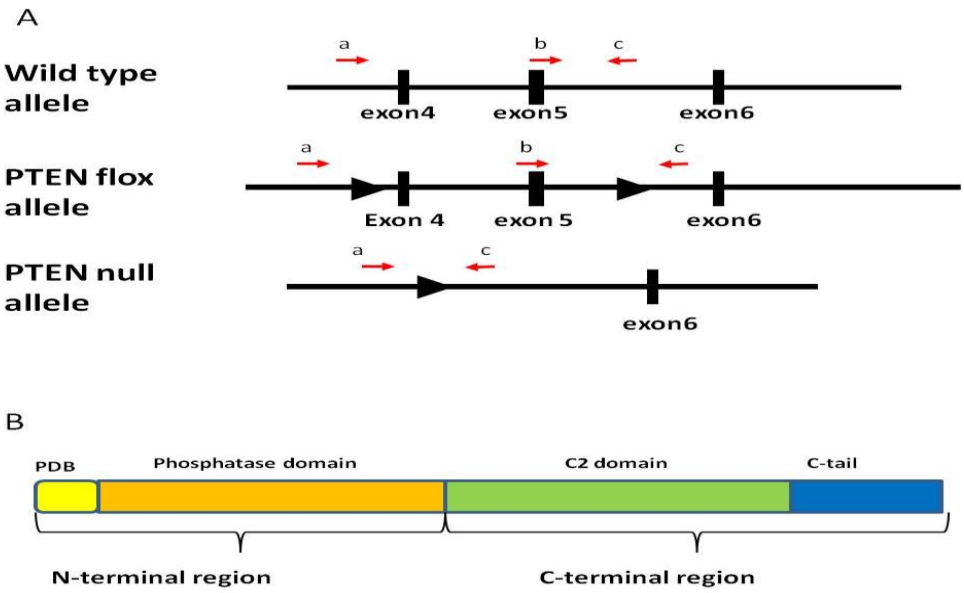


Figure 2.1: PTEN conditional knock out

(A)Targeting strategy for gene deletion as previously described. Black boxes represented exons and the triangles represent Lox P sites. The position of the genotyping primers is represented by red arrows labeled a,b,c. (B) Schematic diagram showing the domains of the PTEN protein. The N-terminal region comprises of a PIP2-binding domain (PBD) and a phosphatase domain whereas the C-terminal consists of the C_2 domain and C-tail.

2.4 PDK1$^{fl/fl}$ Rosa-26cre-ERT and PDK1$^{fl/fl}$ Vav cre

We generated conditional PDK1 knockout mice using the Rosa-26cre-ERT system as well as the Vav-cre system. In the PDK1$^{fl/fl}$ mice, exon 3 and 4 (Fig 2.2A) which encode the kinase domain (Fig 2.2B) were flanked by loxP sites. PDK1$^{fl/fl}$ females were crossed with males that carry a single Rosa-26cre-ERT or Vav-cre recombinase to generate first generation progeny PDK1$^{fl/wt}$ Cre +. PDK1$^{fl/fl}$ Cre+ progeny were achievable on second generation and subsequent generation by mating PDK1$^{fl/fl}$ with PDK1$^{fl/wt}$ Cre+. Both mouse lines were born without any physical deformation. For the detection of PDK1 at DNA level, DNA extracts from ear biopsy and blood were amplified by PCR. The presence of the wild-type and floxed alleles was detected using two primers, forward primer (p99), 5′-ATCCCAAGTTACTGA GTTGTGTTGGAAG-3′; reverse primer (p100r), 5′-TGTGGACAAACAGCAATGAAC ATACACGC-3 producing products of 203bp and 237bp for the wild type and floxed alleles respectively. For detection of the deleted allele, forward primer (p80), 5′-CTATGCTGTGTTACTTCTTGGAGCACAG-3′; and reverse primer (p100r), 5′-TGTGGACAAACAGCAATGAACATACACGC-3′were used producing a 250 bp product. The following PCR programme was used: denaturing at 94ºC for 4 minutes, 35 cycles of 94ºC for 30 seconds annealing at 63ºC for 30 seconds, extension at 72ºC 30 seconds, final annealing at 72ºC for 5 minutes and hold at 4ºC.

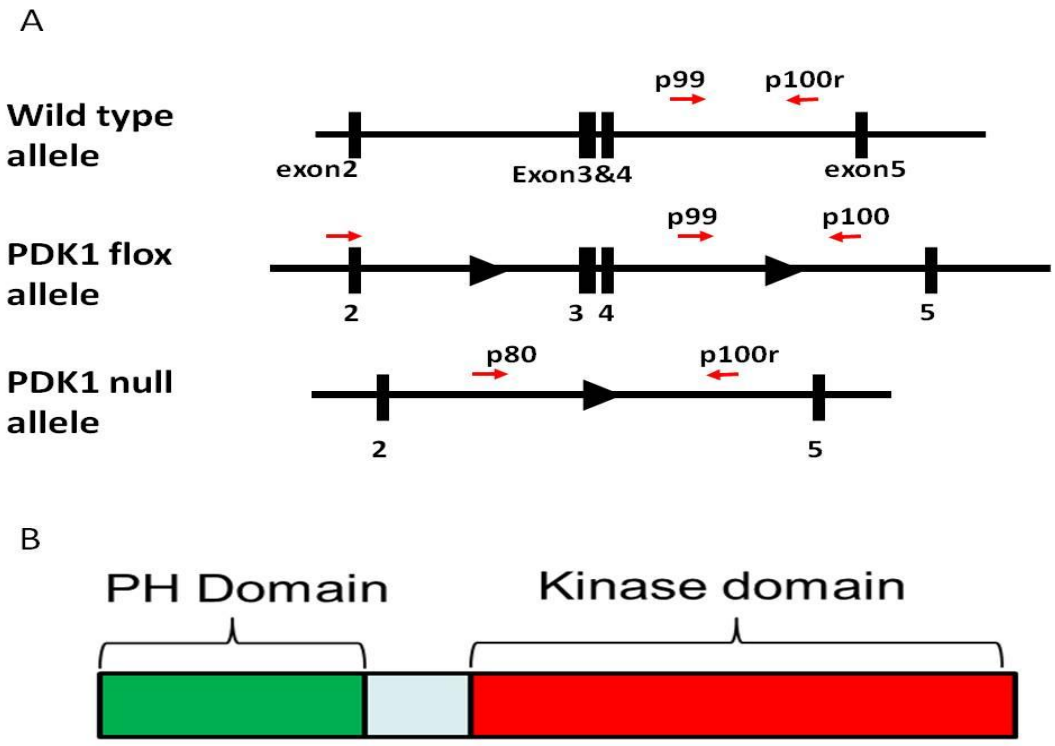


Figure 2.2: PDK1 conditional knock out.

(A) Targeting strategy for PDK1 as previously described. Black boxes represent exons, triangles represent Lox P sites and arrow shows primer position labeled p80, p99 and p100 used for genotyping. (B) Schematic diagram of PDK1 protein showing the PH domain in green and kinase domain in red.

2.5 PDK1$^{L155/L155}$ Rosa-26cre-ERT

The PDK1 L155E mutant is a conditional knock-in, in which the PIF pocket site of PDK1 is disrupted by mutation of Leu-155 to Glu after Cre recombination (Bayascas et al., 2006). The straight, unconditional PDK1$^{L155/L155}$ knock-in is embryonic lethal (Cordon-Barris et al., 2016) and therefore the conditional knock-in was generated using the minigene approach which allowed the mice to express the wild-type PDK1 until cre activation. The minigene containing PDK1 exons 3 to 14 and a neomycin resistance cassette is inserted between exons 2 and 3 whereas the mutation of L155 to Glu is in exon 4 (Fig 2.3). The minigene is flanked by loxP sites and will be deleted

upon cre recombinase activation resulting in the expression of mutant PDK1. These mice were generated using the Rosa-26cre-ERT as well as the Vav-cre system. $PDK1^{L155/L155}$ females were crossed with Rosa-26cre-ERT for the detection of the wild type allele and floxed allele we used two primers, forward primer Primers P1 (5'-GGAATACTCTGTAGACCAGGCTG-3') and P2 (GACGTGTCCTAATACTACCACAAGTGGC-3') producing a 171 bp product for the wild type and 212 bp for the L155E allele. The following PCR programme was used: denaturing at 94ºC for 4 minutes, 35 cycles of 94ºC for 30 seconds annealing at 63ºC for 30 seconds, extension at 72ºC 30 seconds, final annealing at 72ºC for 5 minutes and hold at 4ºC. Using these primers could not confirm the excision of the minigene. Therefore, our alternative approach to determine the excision of the minigene was to use primers that detect to the neomycin resistance cassette which is coupled to the minigene. However, the detection of the neomycin cassette was achieved but the results remained inconclusive as the mice were crossed with Rosa-26cre-ERT mice which also contain a neomycin cassette. DNA isolated from the bone marrow of leukaemic mice was sent to Transnetyx INC laboratories for genotyping to confirm excision of the minigene.

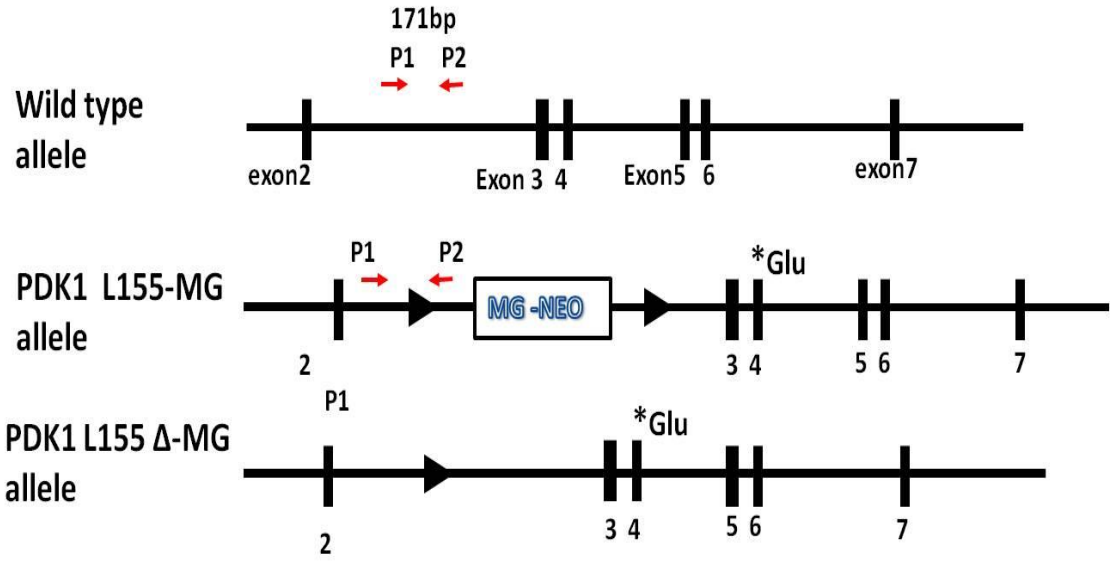


Figure 2.3: PDK1$^{L155/155}$ mutant

Targeting strategy for the L155E mutant as previously described (Collins et al., 2003). The minigene with neomycin cassette sequence is flanked by Lox p sites and is excised during Cre-mediated recombination. An asterisk represents the position of the L155 mutation on exon. Black boxes represent exons, triangles represent Lox P sites and the red arrows shows primer position labeled P1, P2 used for genotyping The wild type allele produced and171 bp fragment whereas the PDK1L55MG produce a 212 bp product.

2.6 PDK1$^{MGK465/MGK465}$

The mutant PDK1 MGK465E knock-in mice were also bred and maintained in the C57BL6 background. The PDK1 MGK465E knock-in was generated by mutation of Lys465 to a Glu residue. The mutation is in exon 12 which encodes for the PH domain of the PDK1 protein (Fig 2.4). This mutation is not embryonic lethal and the mice are born without any defects except for their small body size. We crossed PDK1$^{MGK465E/+}$ with another PDK1$^{MGK45E/+}$ to obtain homozygous mutants. For the detection of the mutant allele at DNA level, genomic DNA was extracted from ear biopsy and blood samples and subjected to PCR with the following primers: Forward primer (5′-GGGTGAAGCATGGAATCTGTGTCTT-3′) reverse (5′GCCAGGATACCTAAGAGTACCTAGAA) producing a 196 bp product for the

wild type and 236 bp for the mutated allele. The following PCR programme was used: denaturing at 94ºC for 4 minutes, 35 cycles of 94ºC for 30 seconds annealing at 63ºC for 30 seconds, extension at 72ºC for 30 minutes, final annealing at 72ºC for 5 minutes and hold at 4ºC.

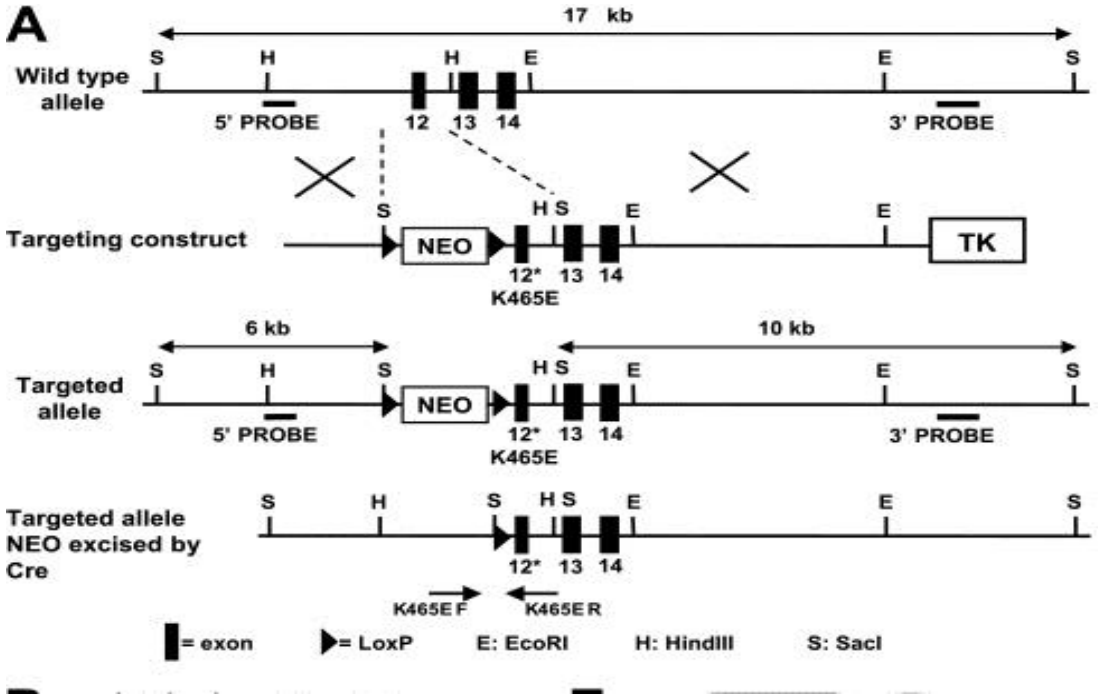


Figure 2.4: PDK1 PDK1$^{MGK465/MGK465}$ mutant

(A) Targeting strategy for the MGK465 mutation as described before (Bayascas et al., 2008) Diagram depicting the 3′ end of the endogenous PDK1 gene from exons 12 to 14, the targeting construct generated, the targeted allele with the neomycin selection cassette still present (NEO), and the targeted allele with the neomycin cassette removed by Cre recombinase. The knock-in allele containing the Lys465Glu mutation in exon 12 is marked with an asterisk and can be detected by genotyping using PCR primers K465E F and K465E R, which are depicted as arrows.

2.7 Breeding strategies for compound knockout

We generated conditional compound knockout PTEN$^{fl/fl}$ PDK1$^{fl/fl}$ with Rosa-26cre-ERT and Vav-cre. The compound knockout PTEN $^{fl/fl}$PDK1$^{fl/fl}$ Rosa-26cre-ERT was generated by crossing PTEN $^{fl/fl}$ Rosa-26cre-ERT males with PDK1$^{fl/fl}$ females without cre. First generation pups where expected to be all heterozygous for PTEN

and PDK1 with 50% of the pups carrying the Rosa-26cre-ERT allele. Second generation progeny was generated by crossing a male with cre and a female without cre. PTEN $^{fl/fl}$-PDK1 $^{fl/fl}$ Rosa-26cre-ERT can be obtained from third generation progeny. The conditional compound knockout mice were born viable without any physical defects. This mouse line was maintained by crossing PTEN$^{fl/fl}$-PDK1$^{fl/fl}$ Rosa-26cre-ERT males with PTEN$^{fl/fl}$-PDK1$^{fl/fl}$ females without cre. To generate the compound knockout mice on Vav-Cre we crossed PTEN$^{fl/fl}$ females without cre with PDK1$^{fl/fl}$ Vav-cre males. First generation pups were expected to be all heterozygous with 50% of the pups carrying the Vav-cre allele. Second generation progeny was generated by crossing a male with cre and a female without Cre. When breeding for the compound knock out with the Vav-cre system it was imperative that the mouse that carries the Cre was a male because a recombined gene resulting from constitutive expression of the Vav-cre could cause adverse effects and lead to death at an early age in mice. Thus, in a breeding pair of mice, only the male but not the female giving birth to the offspring would be affected. The compound Vav-cre line was maintained by breeding heterozygous males with Vav-cre and homozygous females without cre. To generate the compound PDK1 mutant with conditional PTEN knockout we used a similar approach to that used to generate PTEN$^{fl/fl}$ PDK1$^{fl/fl}$ Rosa-26cre-ERT. PDK$^{L155E/L155E}$ or PDK1$^{MGK465/MGK465}$ females were crossed with PTEN$^{fl/fl}$ Rosa-26cre-ERT. PDK1$^{L155E/L155E}$ PTEN$^{fl/fl}$ Rosa-26cre-ERT and PDK1$^{MGK465/MGK465}$ PTEN$^{fl/fl}$ Rosa-26cre-ERT mice could be generated on third generation and were maintained with a similar strategy used to maintain PTEN$^{fl/fl}$ PDK1$^{fl/fl}$ Rosa-26cre-ERT.

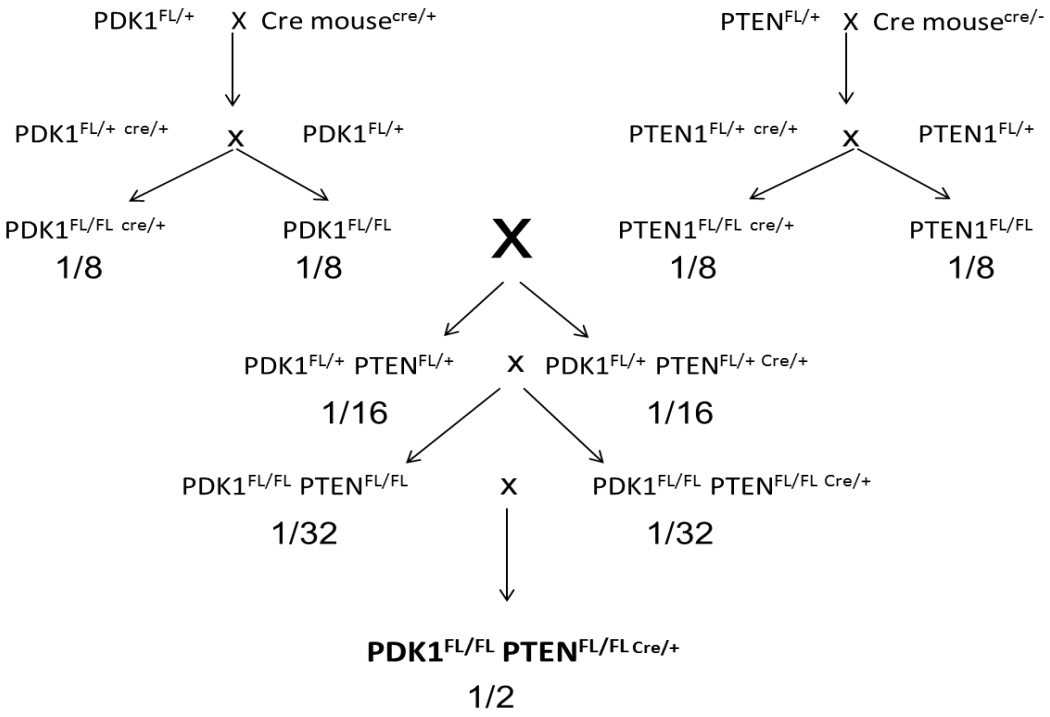


Figure 2.5: Breeding strategy for PTEN$^{fl/fl}$ PDK1$^{fl/fl}$ Rosa-26cre-ERT compound knockout

The compound knockout PTEN$^{fl/fl}$PDK1$^{fl/fl}$ Rosa-26cre-ERT was generated by crossing PTEN$^{fl/fl}$ Rosa-26cre-ERT males with PDK1$^{fl/fl}$ females without cre

2.8 Isolation of Genomic DNA and Genotyping

An ear biopsy was obtained for adult mice, whereas a small piece of the tail or lower extremities of the embryo was cut and placed into 1.5 ml centrifuge tubes. For isolation of genomic DNA we used the Hot SHOT method. Depending on the size of the tissue, about 50 to 75 µl of the Hot SHOT alkaline lyses buffer (Sodium hydroxide solution pH 12) was added to each tube containing the biopsy. To disintegrate the tissue pieces, the tubes were incubated at 95ºC for 15 minutes. An equal amount of neutralising buffer (40 mM Tris-HCL solution pH 5.0) was added to adjust the pH. At this point, the DNA was ready for use with our PCR protocols.

2.9 Genotyping (General protocol)

As a reference 2 µl of DNA was used for each 20 µl PCR reaction contained 1X Dream Green buffer supplemented with 2.5 mM MgCl2 (Fermentas), 0.25 mM dNTP mix (Roche), 1µM from each primer and 0.5 unit of Dream TaqTM Green DNA polymerase (fermentas). Thirty five cycles of amplification were performed.

2.10 Competitive Bone-marrow Transplantation

In order to distinguish donor from competitor cells upon transplantation, our transgenic mice (donor) were always C57BL6 CD45.2 whereas the recipient mice were wild type C57BL6 CD45.1 (SJL). We sacrificed $PTEN^{fl/fl}$ Rosa-cre-ERT26ER, $PDK^{fl/fl}$ Rosa-cre-ERT26ER, $PDK1^{L155E/L155E}$ Rosa-cre-ERT26ER, $PDK1^{MGK465/MGK465}$, compound $PTEN^{fl/fl}$ / $PDK^{fl/fl}$ Rosa-cre-ERT26ER,$PDK1^{L155E/L155E}$/$PTEN^{fl/fl}$Rosa-cre-ERT26E, $PDK1^{MGK465/MGK465}$/$PTEN^{fl/fl}$ Rosa-cre-ERT26ER transgenic mice all C57BL6 CD45.2 (donor) and a wild type C57BL6 CD45.1 (rescue cells) by CO_2 euthanization and prepared the hind limbs using forceps and sharp scissors. The muscle was removed from the tibia and femur from both legs by dissection. The bone marrow was flushed out with suspension media (SM; 0.2% sterile filtered foetal calf serum in PBS) using a 21-gauge needle on to a 40 µm nylon cell strainer into a 50ml falcon tube. Any bone marrow aggregates were gently pushed through the cell strainer using a plunger. The cells were pelleted by centrifugation at 1300 rpm for 5 minutes at 4°C. Once the supernatant was removed, the cell pellet was resuspended in 2ml of erythrocyte lysis buffer (10 mM $KHCO_3$, 150 mM NH_4Cl, 0.1 mM EDTA pH 8.0) for each mouse. Cells were

incubated at room temperature for 10 minutes then 10 ml of cold SM was added before the cells were pelleted by centrifugation. Cells were resuspended in 10 ml cold SM and counted using an improved Neubauer Haemocytometer. Each recipient mouse received 1 mio donor (CD45.2) and 0.2 mio rescue (CD45.1) cells in a volume of 150 µl. Transplantation was done by tail vein injection.

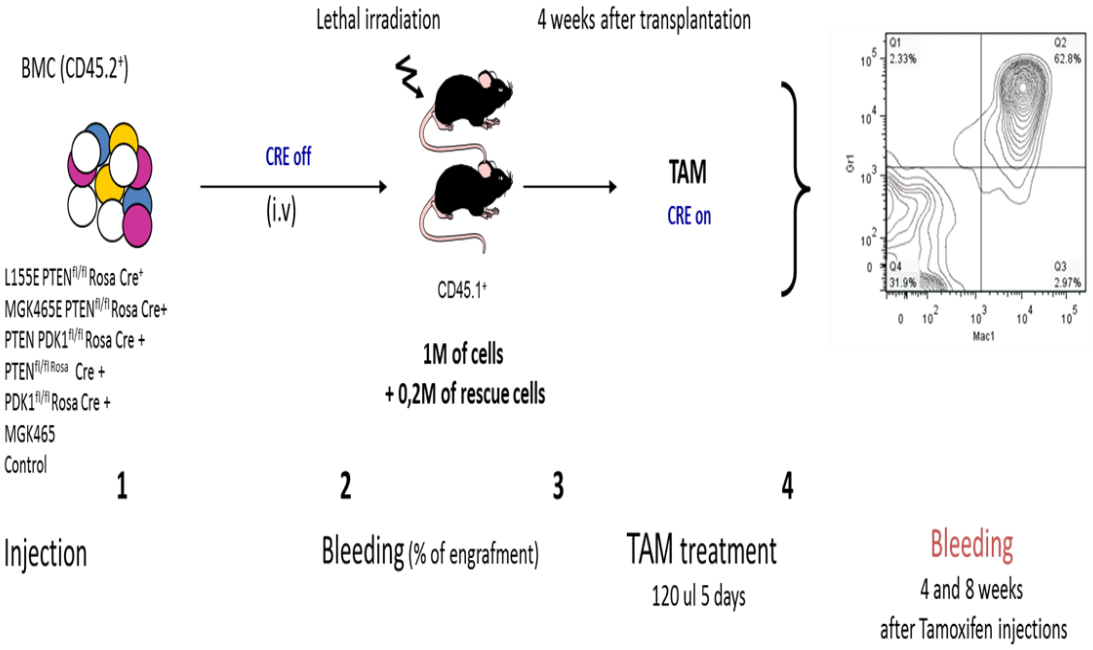


Figure 2.6 Experimental approaches for competitive bone marrow transplant assays

We transplanted the bulk of bone marrow cells (1 Mio cells) from $PTEN^{fl/fl}$ Rosacre26ER, $PDK^{fl/fl}$ Rosa-cre-ERT26ER, $PDK^{L155E/L155E}$ RosaCre26ER, $PDK1^{MGK465/MGK465}$, compound $PTEN^{fl/fl}/PDK^{fl/fl}$ Rosa-cre-ERT26ER,$PDK^{L155E/L155E}/PTEN^{fl/fl}$Rosacre26E, $PDK1^{MGK465/MGK465}/PTEN^{fl/fl}$ RosaCre26ER transgenic mice all C57BL6 CD45.2 together with 0.2 Mio cells from wild type SJL mouse (CD45.1) into lethally irradiated mice (CD45.1).

2.11 Blood reconstitution analysis

Peripheral blood was collected from the recipient mice at different time points to analyse the reconstitution of blood post-transplantation and after treatment with tamoxifen (i.e. 4 weeks post- transplant and 4, 8 and 12 weeks after tamoxifen

treatment). Blood was drawn from tail vein by punching the vein with a needle. Blood was collected into eppendorf tubes pre-coated with 0.01 M Ethylenediaminetetraacetic acid (EDTA) (Sigma). A full blood count was performed using a full blood analyser (Mythic 18 Vet). For preparation of samples for FACS, blood samples were subjected to red cell lysis as previously described (in 2.10). Samples were transferred to BD test tubes for staining.

2.12 General stain

All antibodies used were purchased from Biolegend. Antibodies used were CD45.1 (FITC cat# 110706), CD45.2 (APC cat# 109514), c-Kit (PE cat# 105808), Mac1 (PE-Cy7 cat#101216), Gr1 (PerCP/Cy5.5 cat# 108428) , CD4 (Alexa Floor 700 cat# 100430) , CD8 (APC-Cy7 cat#100714) and B220 (Pacific Blue cat# 103277). A master mix was prepared by mixing of the antibodies at a ratio 1:1.5:0.5 (1 µl of CD45.1 + 1.5µl of CD45.2 and 0.5µl for the rest) per sample. Staining was carried out in 100µl reaction in BD FACS tubes. Unstained samples were also prepared as controls. Unstained and stained samples were incubated at 4ºC in the dark for 30 minutes followed by washed with SM then suspended in 200 µl of SM with 1 µg/ml of propidium iodine and analysed by flow cytometry using the LSR II flow cytometer (BD Bioscience).

2.13 Processing of leukemic mice

Mice showing symptoms of disease (i.e. loss of condition, hunched, heavy breathing, etc.) were sacrificed as previously described. Bone, spleen, liver and thymus were collected from the mice by dissection and weighed. The bone marrow was harvested as previously described. The cells from the other organs were harvested by grinding a piece of the organ with a plunger through a strainer and collecting into a 50 ml tube. Each sample was subjected to red cell lysis as previously described. The total bone marrow count was carried out for each mouse. General stain using the previously described antibodies was carried out for each organ. The CD45.1 and the CD45.2 was used to distinguish between the donor cells and the recipient cells.

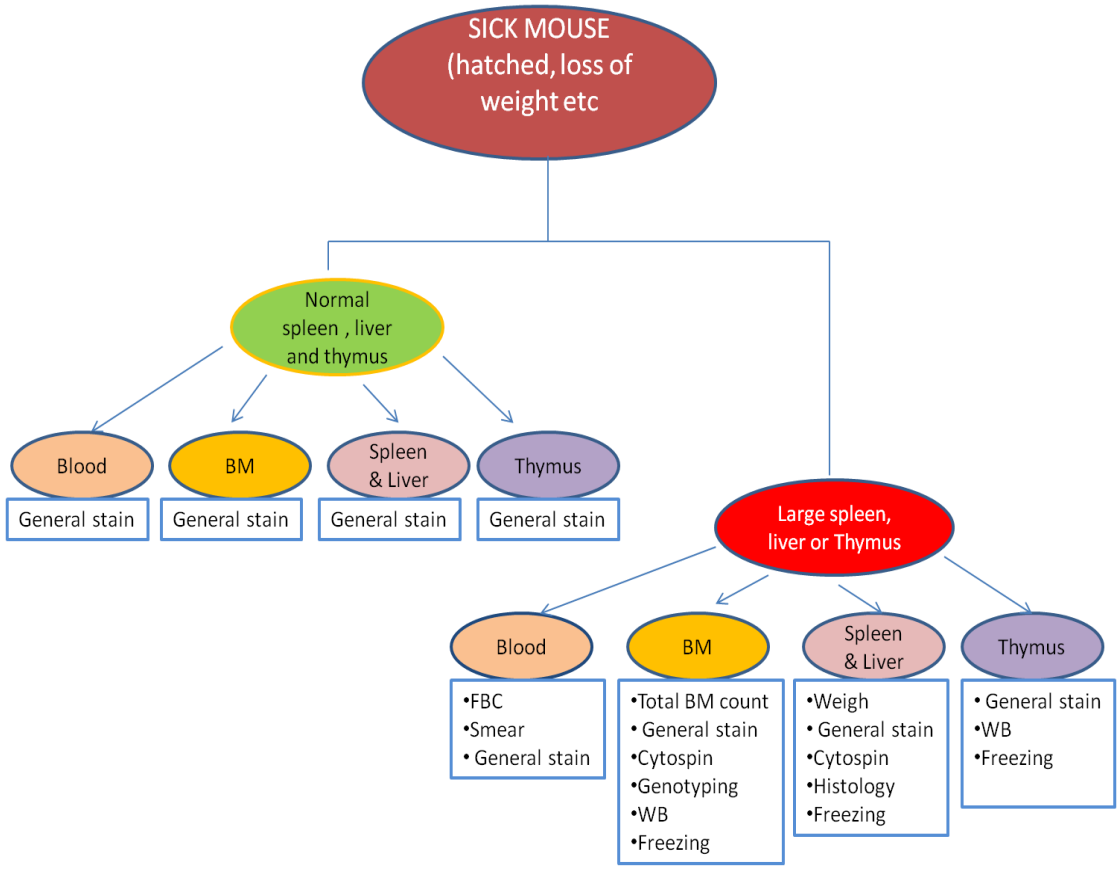


Figure 2. 7 Analysis of sick mouse

2.14 HSC Analysis

HSC analysis was carried out at different time points to determine the reconstitution of the bone marrow post-transplantation and after treatment with tamoxifen (i.e. 4 weeks post-transplant and 4, 8 and 12 weeks after tamoxifen treatment). Three mice from each group at all time points were sacrificed as previously described. Bone marrow cells were collected from the tibia and femur as previously described. Cells were initially stained with primary lineage unconjugated rat IgG (Ter119, Mac1, Gr1, CD3e, CD4, CD8 and B220, Biolegend) at a dilution of 1:40 for each mouse and incubated at 4ºC in the dark for 30 minutes followed by washing with SM. Cells were spun down to a pellet by centrifugation and stained with Texas Red diluted with PBS (1:100) for lineage selection. Following 30 minutes incubation cells were washed and suspended in 70 µl of blocking antibody rat IgG (Sigma) for another 30 minutes at 4ºC. Finally cells were washed with SM then stained with secondary antibodies (Table 2.1) and incubated at 4ºC for 30minutes. Stained Cells were washed and resuspended in SM with 1µg/ml of propidium iodine (PI). HSC analysis was carried out using ARIA cell sorter (BD Bioscience). LSK ($Lin^{-/lo}$ $Sca1^{+}c\text{-}Kit^{+}$), LT-HSC ($Lin^{-/lo}$ $Sca1^{+}c\text{-}Kit^{+}CD150^{+}CD48^{-}$), ST-HSC ($Lin^{-/lo}$ $Sca1^{+}c\text{-}Kit^{+}CD150^{-}CD48^{-}$), LMPP ($Lin^{-/lo}$ $Sca1^{+}c\text{-}Kit^{+}CD150^{-}CD48^{+}$), CMP ($Lin^{-/lo}Sca1^{-}c\text{-}Kit^{+}CD34^{+}CD16/32^{lo}$), GMP ($Lin^{-/lo}$ $Sca1^{-}c\text{-}Kit^{+}CD34^{+}CD16/32^{hi}$) and MEP ($Lin^{-/lo}Sca1^{-}c\text{-}Kit^{+}CD34^{-}CD16/32^{lo}$) .

Table 2.1 Secondary antibodies and their dilutions that used to stain haematopoietic stem/progenitor cells for sorting.

Antibody	Manufacturer	Clone No.	Isotype	Dilution
Anti CD34-FITC	E-Bioscience	RAM 34	Rat IgG_{2a}, κ	1:50
Anti CD16/32-APC	BD Pharmingen	2.4G2	Rat IgG_{2b}, κ	1:75
Anti Ly6A/E (Sca1) PE-Cy7	E-Bioscience	D7	Rat IgG_{2a}, κ	1 :50
Anti CD117 (c-Kit) PE	Biolegend	2B8	Rat IgG_{2b}, κ	1 :200
Anti CD150 Brillant Violet	Biolegend	TC15-12F12.2	Rat IgGa, λ	1 :25
Anti CD48 Alexa Fluor® 700	Biolegend	HM48-1	Armenian Hamster IgG	1 :100

2. 15 Cell Cycle Analysis using Propidium Iodide (PI)

0.1-1x10^6 cells were washed twice with cold PBS in FACS tubes and centrifuged for 5 minutes at 300 g. The supernatant was aspirated and cells were resuspended in 200 µl of PBS and fixed by 70% cold ethanol for at least 1 hour at 4ºC. The fixed cells washed twice with cold PBS and stained with 200 µl of PI stain (PBS: 0.2X FBS, 40ug/ml RNase (Sigma), 40 µg/ml PI (Sigma) for 30 minutes at 4ºC in a dark place. The stained cells were analysed using LSRII flow cytometer.

2.16 HSC and Progenitors Sorting

Mice were sacrificed as previously described. Bone marrow cells were collected from the tibia and femur as previously described. Bone marrow cells were isolated from five wild type C57BL6 CD45.2 mice or transgenic C57BL6 CD45.2 mice after treatment with tamoxifen for 5 days to induce gene deletion. Pooled cells from the five mice were initially stained with primary lineage unconjugated rat IgG (Ter119, Mac1, Gr1, CD3e, CD4, CD8 and B220, Biolegend) at a dilution of 1:40 and incubated at 4ºC in for 30 minutes followed by washing with SM. Cells were incubated with washed Dynabeads (sheep anti-rat IgG, Dynal, Invitrogen, 120.21D) in a 4 ml suspension for 30 minutes at 4ºC on a rotating platform. After incubation, a magnet was applied to the cells and beads mixture for 3 minutes. The unbound lineage negative cells were transferred to a fresh 10 ml test tube. SM was added to the beads again to repeat the separation of unbound linage negative cells from the beads. Cells were spun down to a pellet by centrifugation and transferred to a BD tube for staining with Texas Red diluted in PBS (1:100) to exclude cells that escaped lineage depletion. Following 30 minutes incubation, cells were washed and suspended in 250 µl of blocking antibody rat IgG (Sigma) for another 30 minutes at 4ºC. Finally cells were washed with SM and stained with secondary antibodies (Table 2.1) and incubated at 4ºC for 30minutes. Stained Cells were washed and resuspended in SM with 1 µg/ml of propidium iodine (PI), and then subjected to sort, LT-HSC ($Lin^{-/lo}Sca1^{+}c\text{-}Kit^{+}CD150^{+}CD48^{-}$), LMPP ($Lin^{-/lo}Sca1^{+}c\text{-}Kit^{+}CD150^{-}CD48^{+}$), CMP ($Lin^{-/lo}Sca1^{-}c\text{-}Kit^{+}CD34^{+}CD16/32^{lo}$), GMP ($Lin^{-/lo}Sca1^{-}c\text{-}Kit^{+}CD34^{+}CD16/32^{hi}$) and MEP ($Lin^{-/lo}Sca1^{-}c\text{-}Kit^{+}CD34^{-}CD16/32^{lo}$) using ARIA cell sorter (BD Bioscience).

2.17 Giemsa Stain

Blood was drawn from tail vein or heart puncture to make peripheral blood smears. Bone marrow, spleen and liver cytospins were prepared in a Cytospin™ 4 Cytocentrifuge (Thermo Scientific™) at 300 rpm for 5 minutes. Staining was carried out with May-Grünwald stain (Sigma) for 3 minutes, washed with water followed by stained with Giemsa stain (1:20, Sigma) for 20 minutes. Slides were left to dry for several minutes and examined under a microscope equipped with a camera.

2.18 Histology

Biopsy from spleen and liver were fixed in 10% formalin prior to be sent to Propath (UK) Ltd histology lab. All samples were paraffin embedded and stained using Haematoxylin and Eosin stain (H&E).

2.19 Generation of Mouse embryonic fibroblast (MEF)

All the mice used were fl/fl for the desired allele and one animal in each mating pair also expressed the Rosa-26cre-ERT. Timed mating was set for each mouse line and sexual activity was monitored the next morning by checking the vaginal plugs which composed of coagulated secretions from the male. This was counted as embryonic day 0.5 (E 0.5). Mouse embryonic fibroblasts were harvested at day 13-14.5 of pregnancy. Mice were sacrificed by increasing CO_2 levels until rigor mortis occurred. Uterine horns were removed from the mice by dissection and placed in 4°C cooled Phosphate buffer saline (PBS). The uterine membrane, placenta and surrounding embryo sac

was removed and each embryo was placed in a well of a 6 well plate filled with PBS. The head and tail of the embryo were cut off and used for genomic DNA isolation which was used for genotyping each embryo. The fetal liver as well as all the vital organs were removed using a pair of forceps. The embryos were then transferred into new 6 well plates with cold PBS and grinded using a glass slide into fine slurry of tissue that was collected into 15 ml tube and span down at 1300 rpm for 5 minutes. After removing the supernatant, 2 ml of trypsin (tissue dissociation grade) was added to each tube containing the embryo followed by incubation for 10 minutes at 37ºC with vortexing every 2 minutes. Medium containing 10% FBS was used to deactivate the trypsin followed by another spin at 1300 rpm for 5 minutes. Each embryo was cultured in T175 flask.

2.20 Cell cultures

MEFs were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovin serum. Each flask was coated with 0.1% Gelatin (extract from animal collagen that helps the cells to adhere to the flask). Medium was replaced or replenished when the colour changed to yellow before cultured cells grew too dense. Cell lines were frozen using freezing medium (90% FCS and 10% DMSO) and stored in -80°C or in liquid nitrogen after 3 to 5 passages.

Primary cells from leukaemic mice were cultured in R20/20 (RPMI1640, 20% FCS, 20% WEHI, 100 U/ml penicillin, and 100 μg/ml streptomycin). WEHI is a conditioned medium (source of IL-3). Medium was replaced or replenished when the

colour changed to yellow before cultured cells grew too dense. Cell lines were frozen using freezing medium (90% FCS and 10% DMSO) and stored in -80°C or in liquid nitrogen after 3 to 5 passages.

2.21 PI3K/PDK1/AKT/PKC Inhibitors

MEFs were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovin serum in a six well plate (3x 10^5 cells per well). Medium was removed next and replaced with fresh medium. Cells were treated for 60 minutes with increasing dose (0.5 µM to 10µM) of PI3K inhibitor (LY294002 cat# s1105 selleckchem), PDK1 inhibitor (GSK2334470 cat# s7087 selleckchem) AKT inhibitor (AKTVIII gift from Dr. Maria Teresa) and PKC inhibitor (PKC817 gift from Prof. Peter Parker). Cells were rinsed 3 x with PBS and extracted for western blot analysis.

2.22 Western Blotting

Immunoblotting was used to confirm the deletion of PTEN and PDK1 as well as to investigate the phosphorylation of PDK1 substrates. Cells in culture plate were washed with cold PBS after aspiration of the culture medium. PBS was aspirated and ice cold NP40 buffer containing Tris-HCL 100 mM, pH 7.4, NaCL 300 mM, NP-40 2% and EDTA 10 mM was added to the cells then left to lyse for 30 minutes on ice. For reference, we used 150 µl of lysis buffer per well in a 6 well plate. The adherent cells were scraped off the plate using a plastic cell scraper and gently transferred into pre-cold micro-centrifuge tubes then centrifuged at 16000 g for 20 minutes at 4ºC.

The supernatant contained the proteins of interest and at this point aliquots were taken and frozen down while at the same time 10 µl was also taken for protein quantification. Proteins were quantified for each sample using the Pierce 660 nm Protein solution. The reagent and the sample were equilibrated to room temperature. Sample and reagent were mixed at a ratio 1:15 in a 96 well micro plate and incubated at room temperature for 5 minutes. A zero reference was prepared by mixing the lysis buffer with the reagent at the same ratio. The absorbance of the sample was measure using the Nano Drop 8000 against a standard of known concentrations. As a reference we used 20 µg of protein for each western blot.

Samples were mixed with 2% SDS buffer containing 0.5M Tris pH 6.8, 50% Glycerol, 10% SDS and bromophenol blue (ratio 1:1). Beta mercaptoethanol was added freshly to the buffer and samples were heated to 95ºC for 5 minutes. SDS Page electrophoresis was run with 10% gradient SDS polyacrylamide gel (NEXT GEL™) at 140V for 60 minutes followed by a blotting of the separated proteins onto nitrocellulose membrane (Amersham ™ Hybond ™ ECL) at 110v for 90 minutes. Blocking was carried out using 5% milk in 1% Tween – PBS for 1 hour and the membrane was subsequently probed with primary antibodies. Rabbit anti mouse antibodies used were purchased from Cell Signalling Technologies. These included PDK1 (cat # 5662S), PTEN (cat #9559L) phosphor Akt 308 (cat# 4056S) phosphor Akt 473 (cat# 9271S) pan Akt (cat# 4685S). The β-actin conjugated with HRP was purchased from Santa Cruz (cat# 1-19; SC-1616) as well as phosphor SGK1 256 (cat# 16744R). In general, incubation with primary antibody was carried out at 4ºC overnight. Incubation with secondary antibody was done at room temperature for an hour after the primary antibody has been washed off. The secondary antibody was anti rabbit conjugated with peroxidase (Jackson ImmunoResearch Laboratory, INC). In general, washing was done 3 times

with 1% Tween- PBS between after each step for 10 minutes each (blocking, primary antibody, secondary antibody.) Proteins were detected using ECL™ Western Blotting Detection reagent,

2.23 Immunofluorecensce

Prior to cell culture, 22 × 22 mm No. 1.5 cover slips from Thermo Scientific (cat# CBAD00220RA20MNZ) were sterilized with 70% ethanol and coated with 0.1% gelatine. MEFs were cultured in a 6 well plate containing the cover slips until they were 90% confluent. Medium was aspirated from the cultures and cells washed with appropriate volume of PBS. Cells were fixed for 15 minutes by adding 4% formaldehyde to a depth of 2 to 3 mm to cover the entire well. The fixative was aspirated and cells rinsed three times with PBS for 5 minutes each. Blocking buffer (1x PBS /5% normal serum/0.3% Triton ™ X-100) was added over the cells in each well followed by incubation for 30 minute at room temperature. Primary antibody (phosphor AKT 308 cat# D25E6 pan AKT cat# 40DA from Cell Signalling Technologies) was prepared dilution to 1:200 with antibody dilution buffer (1X PBS/ 1% BSA/0.3% Triton ™ X-100). After incubation, blocking buffer was aspirated and diluted primary antibody was applied. Incubation with primary antibody was carried out at 4^{o}C overnight in a humid light-tight box to prevent drying. Plates were rinsed three times for 5 minutes each with PBS. The fluorochrome-conjugated secondary antibody (ant-rabbit cat# 4412s anti-mouse cat# 4409s from Cell Signalling) diluted 1:200 was applied and plates incubated at room temperature in humid light-tight box to prevent drying fluorochrome fading. Plates were rinsed three times with PBS for 5

minutes each. Cover slips were removed from the plate and mounted on a microscope slide. Slides were stored at 4^{o}C until analysis by fluorescence microscope.

Chapter3 PTEN and PDK1 in normal haematopoiesis

3.1 A brief introduction

I previously discussed the role of various signalling pathways involved in sustaining normal haematopoiesis. For instance, the Wnt and Notch pathways support normal cell proliferation by promoting normal stem cell self-renewal (Molofsky et al. 2004), (Reya et al., 2001). On the other hand, tumour suppressors such as p53, PML and PTEN also regulate cell proliferation. The role of PTEN on normal haematopoiesis has been previously studied and shown to play a fundamental role in maintaining normal haematopoietic stem cells. Yilmaz and colleagues demonstrated that deleting PTEN in bone marrow cells resulted in exhaustion of normal haematopoietic stem cells (Yilmaz et al., 2006b). In this study, they transplanted $Pten^{fl/fl}$; Mx-1-Cre bone marrow cells or wild-type bone marrow cells both CD45.2 into recipient CD45.1 mice along with half as many rescue cells from the bone marrow of a CD45.1 mouse. Recipient mice were treated with pIpC 6 weeks after they showed stable chimaerism.

They demonstrate that 5 to 20 weeks post administration of pIpC, the total number of control Pten $^{fl/+}$; Mx- 1-Cre donor HSCs remained constant over time compared to $PTEN^{fl/fl}$; Mx-1-Cre donor HSCs which initially dominated the HSC pool, but dropped post pIpC treatment. At the end of 20 weeks, the proportion of control PTEN $^{fl/+}$ Mx-1-Cre HSC donor cells remained at 64% of bone marrow population, whereas $PTEN^{fl/fl}$; Mx-1-Cre donor HSC cells decreased to 15% of bone marrow cells (Yilmaz, 2006). This study showed that PTEN deletion leads to the depletion of normal HSCs while at the same time supporting the production of leukaemia-initiating cells. These findings suggest an extraordinary requirement for maintenance of leukaemia-stem

cells that might diverge from requirements for maintenance of normal stem cells. More importantly, they demonstrated that treatment with rapamycin rescued normal HSC function and prolonged the life of PTEN $^{fl/fl}$; Mx-1-Cre mice when PTEN was deleted. The drug rapamycin inhibits the activity of mTOR kinase, which is one of the downstream targets of the PI3K pathway. To gain further insights into the molecular pathway regulating PTEN function in normal haematopoiesis, we decided to study the role of PTEN and PDK1 using a mouse model where we can simultaneously delete PTEN and PDK1 in the haematopoietic compartment.

3.2 Role of PTEN and PDK1 in normal haematopoiesis

To investigate the functional relationship between PDK1 and PTEN during haematopoiesis, we performed bone marrow transplantation experiments using total bone marrow cells isolated from PTEN$^{fl/fl}$ Rosa-26cre-ERT, PTEN$^{fl/fl}$ PDK$^{fl/fl}$ Rosa-26cre-ERT and normal wild type cells from a C57BL6. In order to distinguish donor from competitor cells upon transplantation, our transgenic mice (donor) were all C57BL6 CD45.2 whereas the recipient mice were wild type SJL CD45.1. We transplanted 1 mio donor cells (CD45.2) together with 0.2 mio competitor/rescue cells (CD45.1) into lethally irradiated SJL mice (CD45.1) (n=12 for each group). Four weeks post transplant, we sacrificed 3 mice from each group to determine if the transplant was successful and to perform phenotypic analysis. We show that a stable chimaerism was archived four weeks post transplant as demonstrated by the presence of both CD45.1 (rescue cells) and CD45.2 (donor cells) in the bone marrow spleen and liver. We noticed at 4 weeks post transplant, the proportion of donor cells was

higher in the bone marrow compared to other organs in all mice respective of the cell type transplanted (Fig 3.1 A to C). We also compared the weight of the spleen and liver among mice that received PTEN$^{fl/fl}$ Rosa-26cre-ERT, PTEN$^{fl/fl}$ PDK1$^{fl/fl}$ Rosa-26cre-ERT and normal wild type cells. We observed no significant difference in weight 4 weeks post transplant (Fig 3.1 D and E).

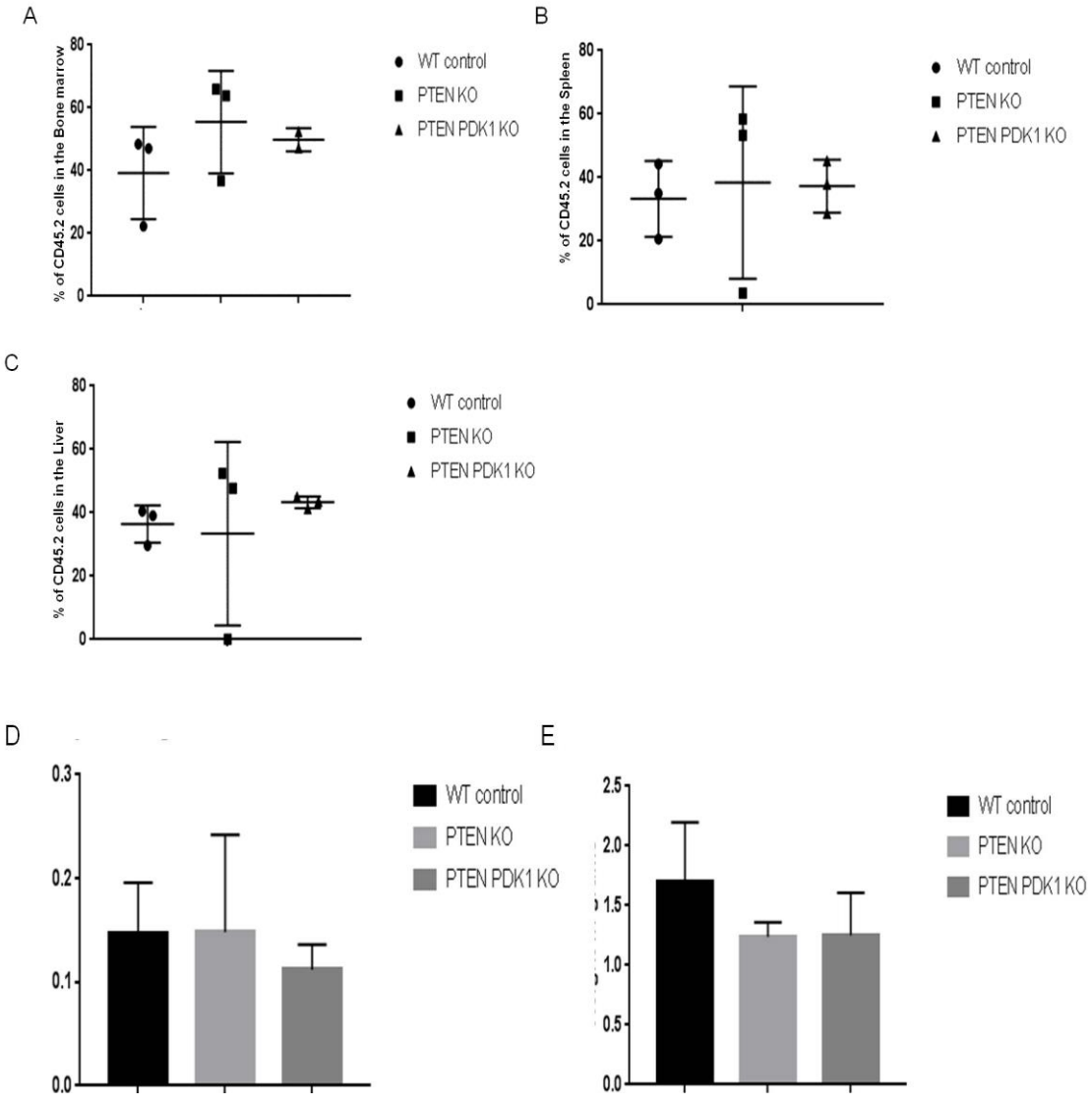


Figure 3.1 Engraftment of PTEN$^{fl/fl}$ Rosa-26cre-ERT and PTEN $^{fl/fl}$PDK1$^{fl/fl}$ Rosa-26cre-ERT cells four weeks after transplant.

Total bone marrow cells from PTEN$^{fl/fl}$ PDK1$^{fl/fl}$ Rosa-26cre-ERT mouse (1 Mio CD45.2 cells) were transplanted into lethally irradiated SJL mice (CD45.1 n=10) and 0.2 Mio CD45.1 cells used for rescue. (A) Engraftment of donor cells in the bone marrow. (B) Engraftment of donor cells in the spleen (C)

Engraftment of donor cells in the liver at four weeks post transplantation. (D and E) weight of the spleen and liver respectively.

3.2.1 Multi lineage reconstitution with donor cells

To determine the ability of PTEN$^{fl/fl}$ Rosa-26cre-ERT, PTEN$^{fl/fl}$ PDK1$^{fl/fl}$ Rosa-26cre-ERT and normal wild type cells to fully reconstitute. Recipient mice were sacrificed 4 weeks post-transplant and analysed by FACS. Using a panel of antibodies specifically against myeloid markers Mac1, Gr1, stem and progenitor cell marker c-Kit, lymphoid markers CD4, CD8 and B220, as well as CD45.2 and CD45.1, we were able to distinguish the donor cells from rescue cells and also assess the lineage distribution of haematopoietic cells originating from the donor. We demonstrate that a full multi-lineage reconstitution of the bone marrow was achieved in all groups and the lineage distribution was comparable in all groups. The double positive Mac1 and Gr1 population was slightly higher but not statistically significant in mice that received PTEN$^{fl/fl}$ Rosa-26cre-ERT and PTEN$^{fl/fl}$ PDK1$^{fl/fl}$ Rosa-26cre-ERT cells respectively (Fig 3.2). We also noticed a slightly higher but not significant percentage of B220 positive cells in mice that received wild type cells compared to the other groups.

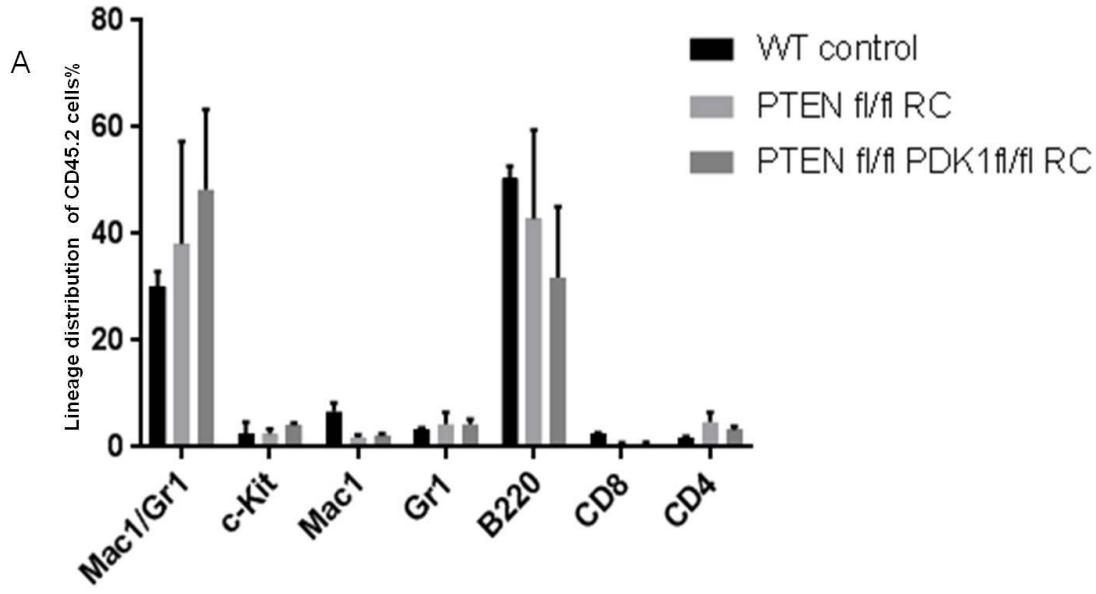


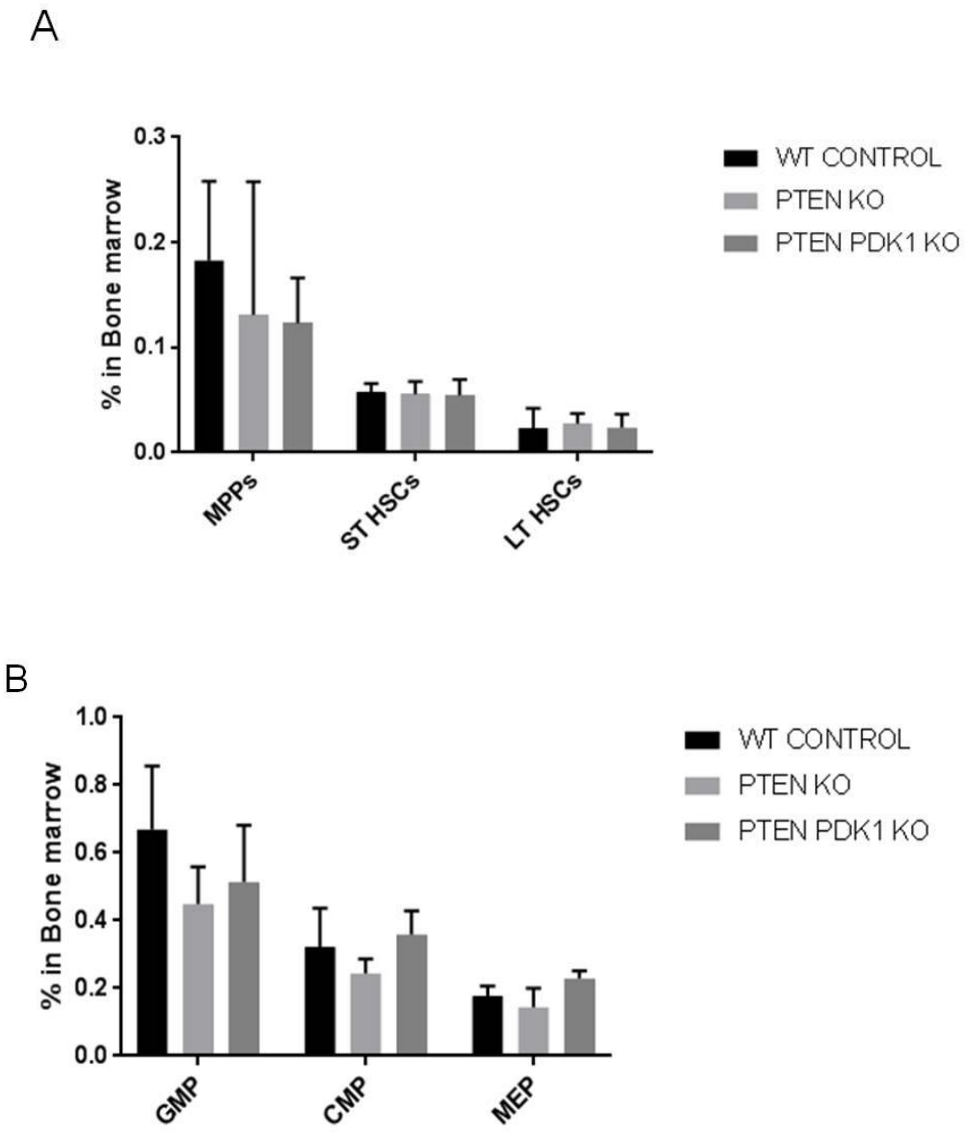
Figure 3.2 Reconstitution with PTEN$^{fl/fl}$ Rosa-26cre-ERT and $^{PTENfl/fl}$PDK1$^{fl/fl}$ Rosa-26cre-ERT cells four weeks after transplant.

Multi lineage reconstitution of the bone marrow was achieved in all groups and the lineage distribution was comparable in all groups. No statistical increase or decrease in the cell lineage was observed in all groups.

3.2.2 Distribution of HSC and early progenitors four weeks after transplant

To further analyse the reconstitution of the bone marrow at four weeks post-transplant, we determined the distribution of HSC and early progenitors in bone marrow using classical LSK and SLAM family markers (CD150 and CD48) (Yeung and So, 2009, Oguro et al., 2013). LT-HSC (Lin $^{-/lo}$Sca1^{+}c-Kit^{+}CD150^{+}CD48^{-}), ST-HSC (Lin$^{-/lo}$Sca1^{+}c-Kit^{+}CD150^{-}CD48^{-}), MPP (Lin$^{-/lo}$Sca1^{+}c-Kit^{+}CD150^{-}CD48^{+}), CMP (Lin$^{-/lo}$Sca1^{-}c-Kit^{+}CD34^{+}CD16/32^{lo}), GMP (Lin$^{-/lo}$Sca1^{-}c-Kit^{+}CD34^{+}CD16/32^{hi}) and MEP (Lin$^{-/lo}$Sca1^{-}c-Kit^{+}CD34^{-}CD16/32^{lo}). The distribution of the HSCs and early progenitors was comparable at this time point (Fig 3.3A and B). However, in general the mice that received wild type cells had slightly higher numbers of MPPs and GMPs

compared to the PTEN$^{fl/fl}$ PDK1$^{fl/fl}$ Rosa-26cre-ERT and PTEN$^{fl/fl}$ Rosa-26cre-ERT group. Of note, the analysis of the HSCs and early progenitors was done on the general bone marrow cells of each mouse without gating for CD45.2 and CD45.1. Given that the earlier analysis of the c-Kit expression in CD45.2 cells gave comparable results across all groups, we conclude that any masking effects of the present competitor cells will be similar in all groups, suggesting no effects in the CD45.2 LSK compartments of the investigated groups. We also analysed the cell cycle of the bone marrow four weeks post-transplant and we found that there was no significant difference in the cell cycle status of the whole bone marrow (Fig 3.3 C).



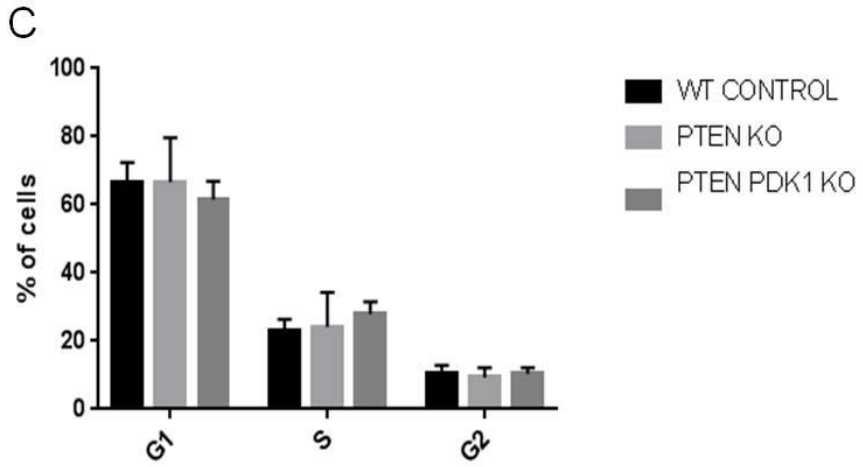


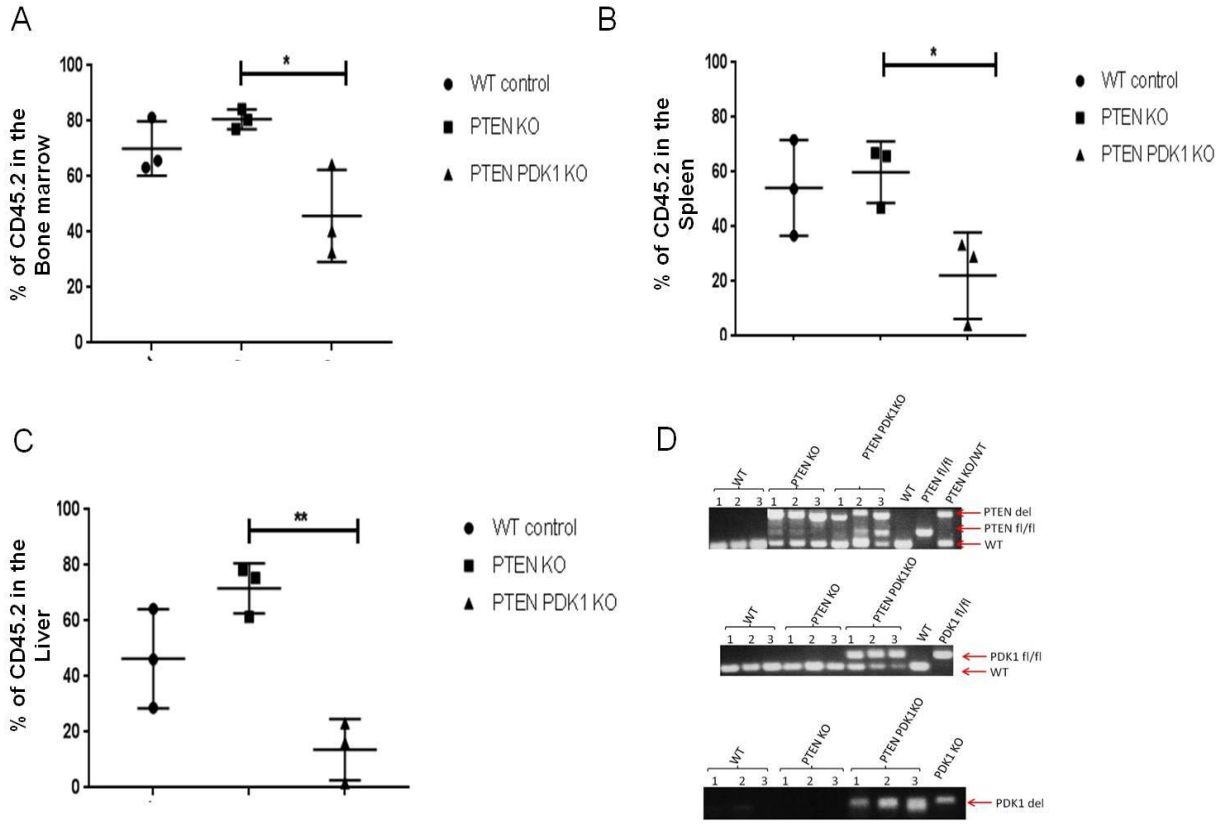
Figure 3.3 Analysis of HSCs and early progenitors four weeks after transplant.

(A) Distribution of LT-HSCs, ST-HSCs and MPPs four weeks after transplant. (B) Distribution of early progenitors GMP, CMP and MEP four weeks after transplant. (C) Cell circle analysis of total bone marrow cells four weeks after transplant.

3.2.3 Impaired engraftment after deletion of PDK1 in PTEN deficient haematopoietic cells.

To investigate the role of PTEN and PDK1, we induced gene deletion by treating the recipient mice with 120ul of tamoxifen for 5 days to activate cre activity. Four weeks post treatment with tamoxifen, we sacrificed three mice from each group. Deletion of PTEN and PDK1 was confirmed by PCR using genomic DNA isolated from the bone marrow of recipient mice (Fig 3.4D). We compared the level of engraftment in the bone marrow, spleen and liver. As expected, the level of engraftment increased in mice that received PTEN $^{fl/fl}$ Rosa-26cre-ERT cells, which was in stark contrast to mice received PTEN$^{fl/fl}$ PDK1$^{fl/fl}$ Rosa-26cre-ERT cells in the bone marrow, spleen and liver (Fig 3.4 A to C). Engraftment of PTEN$^{fl/fl}$ Rosa-26cre-ERT cells was not statistically significantly different as compared to engraftment of wild type cells. We

also compared the weight of the spleen and liver of mice that received PTEN$^{fl/fl}$ Rosa-26cre-ERT cells with mice that received PTEN$^{fl/fl}$ PDK1$^{fl/fl}$ Rosa-26cre-ERT or wild type cells. The spleen weight of mice that received PTEN$^{fl/fl}$ Rosa-26cre-ERT cells were significantly higher compare to spleen weight of mice that received wild type cells and to a lesser extent higher that the spleen weight of mice that received PTEN$^{fl/fl}$ PDK1$^{fl/fl}$ Rosa-26cre-ERT (Fig 3.4 E and F). In overall deletion of PTEN alone resulted in increased proliferation of donor cells in all the organs. On the other hand, deletion of PTEN and PDK1 resulted in decreased engraftment levels of donor cells, suggesting that PDK1 indeed mediates at least part of the loss of PTEN effects.



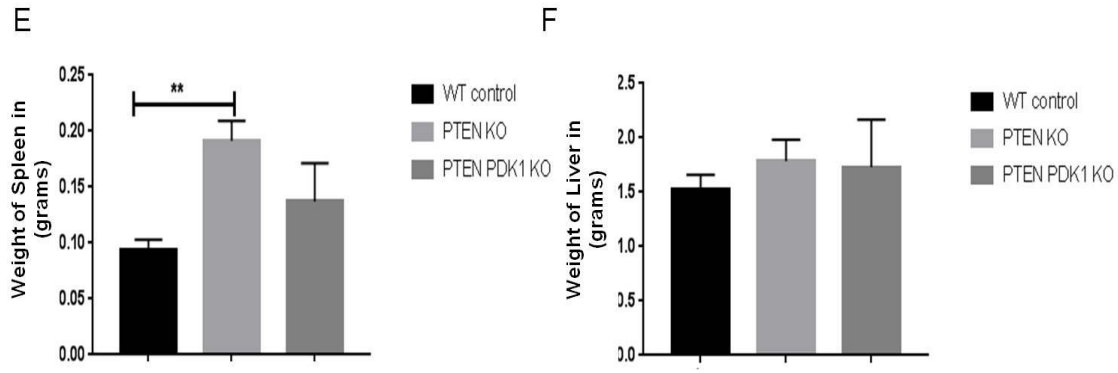


Figure 3.4 Engraftment of PTEN$^{fl/fl}$ Rosa-26cre-ERT and PTEN $^{fl/fl}$PDK1$^{fl/fl}$ Rosa-26cre-ERT cells four weeks post cre activation.

Total bone marrow cells from PTEN$^{fl/fl}$ PDK1$^{fl/fl}$ Rosa-26cre-ERT BM (1Mio cells CD45.2 cells) transplanted lethally irradiated SJL mice (CD45.1 n=10) (0.2 Mio CD45.1 cells used for rescue. (A) Engraftment of donor cells in the bone marrow. (B) Engraftment of donor cells in the spleen (C) Engraftment of donor cells in the liver. (D) PCR confirming deletion of PTEN and PDK1 in the bone marrow (E and F) weight of the spleen and liver respectively. Significance (* P <0.05 ** P >0.005 *** P > 0.001)

3.2.4 PTEN deletion influenced lineage selection

In addition to comparing the engraftment level four weeks post tamoxifen treatment, we analysed the bone marrow to investigate whether the conditional knock out of PTEN and PDK1 has an effect on the different haematopoietic populations' reconstituting the recipient mice. Firstly, we investigated the distribution of mature haematopoietic cells using a panel of antibodies for mature cells lineage markers previously described together with the c-Kit for haematopoietic stem cell and progenitors. Deletion of PTEN enhanced the proliferation of myeloid cells while virtually depleting the B220 cells (Fig 3.5A). Concurrent deletion of PDK1 almost completely reversed these effects in PTEN null mice and showed a comparable pattern to the wild type. We looked at the distribution of the c-Kit positive cells which is a

marker for HSCs and early progenitor within the CD45.2 population (Fig 3.5B). Interestingly, the c-Kit positive population in the mice that received PTEN$^{fl/fl}$ Rosa-26cre-ERT and PTEN$^{fl/fl}$ PDK1$^{fl/fl}$ Rosa-26cre-ERT cells was slightly decreased compared to mice that received wild type cells. However, the reduction was not statistically significant at this time point (Fig 3.5 B).

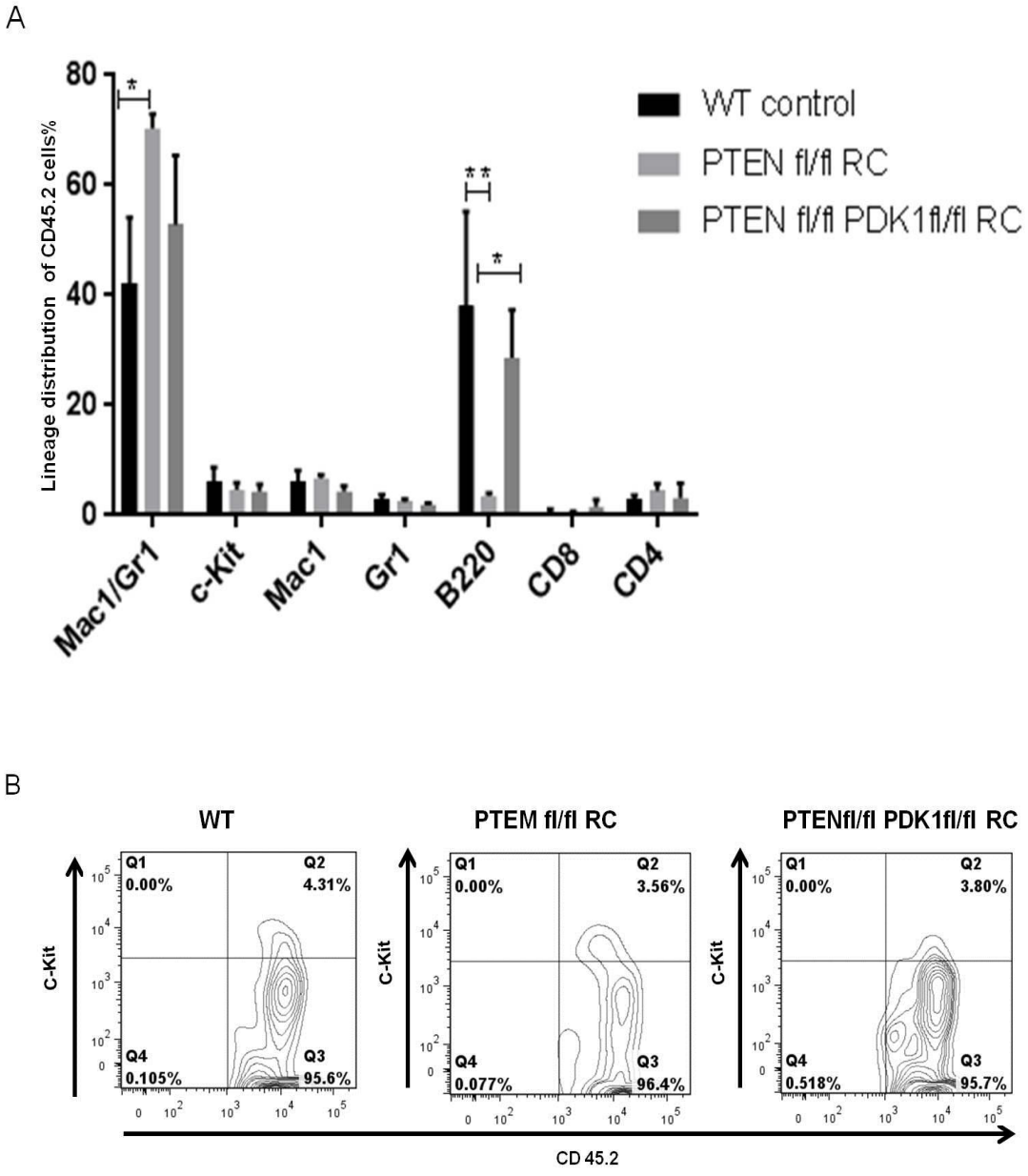


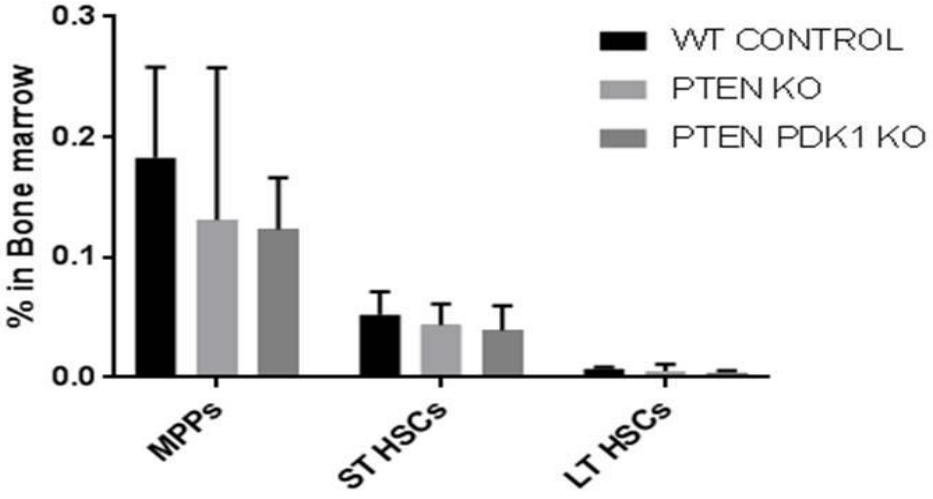
Figure 3.5 Reconstitution with PTEN$^{fl/fl}$ Rosa-26cre-ERT and PTEN $^{fl/fl}$PDK1$^{fl/fl}$ Rosa-26cre-ERT cells four weeks post cre activation

(A) Deletion of PTEN enhanced the proliferation of myeloid cells while virtually depleting the B220 cells whereas deletion of PTEN and PDK1 showed a moderate expansion of myeloid cells and decrease of B220 cells suggesting a mild delay of lineage selection induced by PTEN deletion. (B) Distribution of the c-Kit positive cells within the CD45.2 population. Significance (* P <0.05 ** P >0.005 *** P > 0.001)

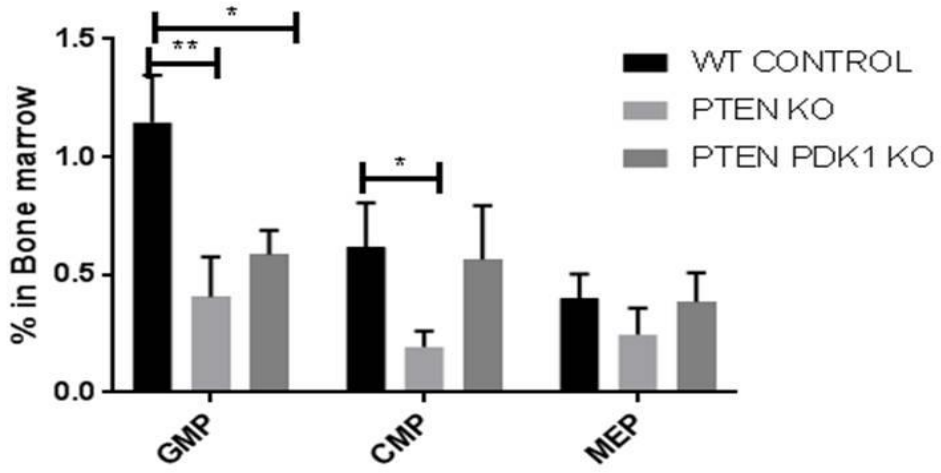
3.2.5 PTEN deletion disturbed normal the distribution of haematopoietic stem cells and progenitors.

To further investigate the effect of PTEN and PDK1 on normal haematopoietic function four weeks post treatment with tamoxifen, we assessed the composition of the bone marrow using a panel of antibodies previously described for the HSCs and early haematopoietic progenitors. Four weeks after tamoxifen treatment, the proportion of LT-HSC ($Lin^{-/lo}Sca1^{+}c\text{-}Kit^{+}CD150^{+}CD48^{-}$), ST-HSCs ($Lin^{-/lo}Sca1^{+}c\text{-}Kit^{+}CD150^{-}CD48^{-}$) and MPP ($Lin^{-/lo}Sca1^{+}c\text{-}Kit^{+}CD150^{-}CD48^{+}$) was not significantly affected in mice transplanted with $PTEN^{fl/fl}$ $PDK1^{fl/fl}$ Rosa-26cre-ERT cells or $PTEN^{fl/fl}$ Rosa-26cre-ERT cells as well as wild type cells (Fig3.6A). Interestingly, the GMP ($Lin^{-/lo}Sca1^{-}c\text{-}Kit^{+}CD34^{+}CD16/32^{hi}$) was significantly reduced in mice that received $PTEN^{fl/fl}$ Rosa-26cre-ERT cells and $PTEN^{fl/fl}$ $PDK1^{fl/fl}$ Rosa-26cre-ERT cells. Similarly, the CMP ($Lin^{-/lo}Sca1^{-}c\text{-}Kit^{+}CD34^{+}CD16/32^{lo}$) was also reduced but only in mice that received $PTEN^{fl/fl}$ Rosa-26cre-ERT cells (Fig 3.6B). Cell cycle distribution did not show any significant disturbance in mice that received either $PTEN^{fl/fl}$ Rosa-26cre-ERT cells or $PTEN^{fl/fl}$ $PDK1^{fl/fl}$ Rosa-26cre-ERT cells (Fig 3.6C).

A


0.3
0.2
0.1
0.0
% in Bone marrow
WT CONTROL
PTEN KO
PTEN PDK1 KO
MPPs
ST HSCs
LT HSCs

B


1.5
1.0
0.5
0.0
% in Bone marrow
*
**
*
WT CONTROL
PTEN KO
PTEN PDK1 KO
GMP
CMP
MEP

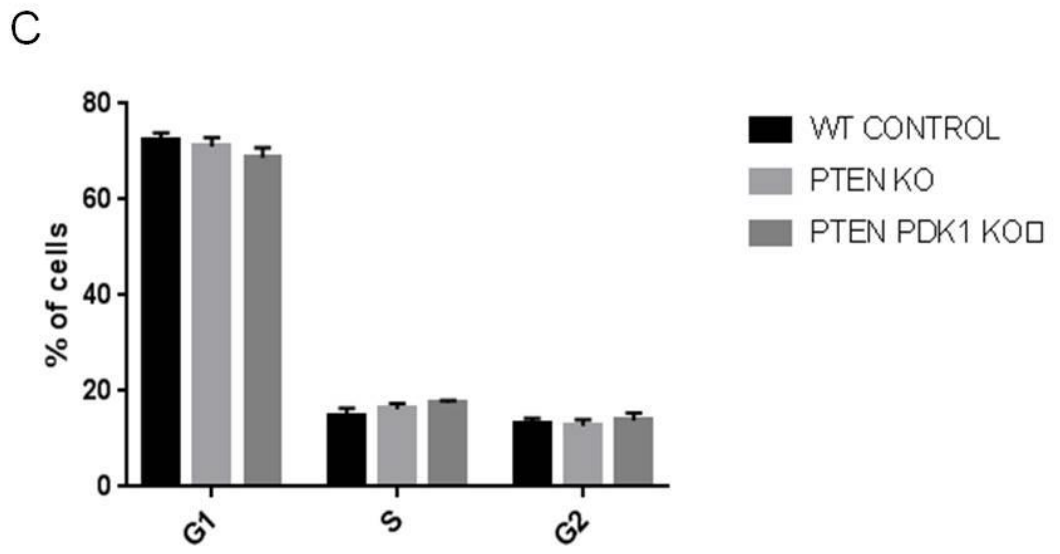


Figure 3.6 Analysis of HSCs and early progenitors eight weeks after transplant

(A) Distribution of LT-HSCs, ST-HSCs and MPPs four weeks after transplant. (B) Distribution of early progenitors GMP, CMP and MEP four weeks after transplant. Cell circle analysis of total bone marrow cells four weeks after transplant. Significance (* P <0.05 ** P >0.005 *** P > 0.001).

3.2.6 PDK1 controls the phosphorylation of AKT 308 in normal haematopoietic cells

To test the effect of PTEN and PDK1 deletion in the in normal hematopoietic cells we analysed the phosphorylation of AKT Thr308, AKT Ser473 and SGK1 Thr256. In parallel with HSC analysis we sorted the donor cells from each of the recipient mice sacrificed four weeks post tamoxifen. Due to the limited amount of cells we obtained after sorting, we grouped cells obtained from mice that received the same type of cells together. As expected, cells isolated from the bone marrow of mice transplanted with $PTEN^{fl/fl}$ Rosa-26cre-ERT had constitutively high levels of phosphorylated AKT at Thr308 and Ser473 as well as SGK1 Thr256 (Fig 3.7). Conversely, we could not

detect phosphorylated AKT Thr308 in cells isolated from mice transplanted with PDK1$^{fl/fl}$ PTEN$^{fl/fl}$ Rosa-26cre-ERT or wild type cells. These result sugget that phosphorylation of AKT Thr308 is mediated by PDK1 in the haematopoietic cells as deletion of PDK1 in PTEN deficient cells was sufficient to completely inhibit the loss of PTEN driven phosphorylation of AKT Thr308 in normal haematopoietic cells.

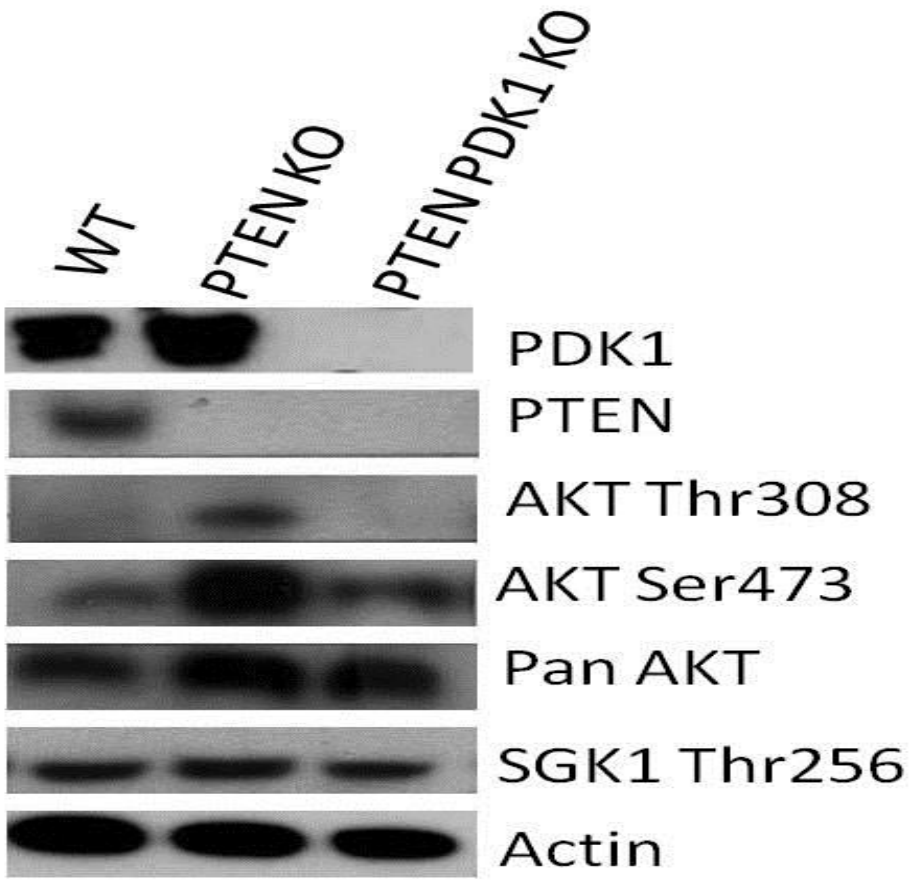


Figure 3.7 PDK1 is required for Phosphorylation of AKT Thr308 in normal haematopoiesis.

Western blot analysis of AKT Thr308, Ser473 and SGK1 Thr256 phosphorylation in PTEN$^{fl/fl}$ Rosa-26cre-ERT and PDK1$^{fl/fl}$ PTEN$^{fl/fl}$ Rosa-26cre-ERT bone marrow cells

3.2.7 Engraftment of PTEN$^{fl/fl}$ PDK1$^{fl/fl}$ Rosa-26cre-ERT is restored 8 weeks after Cre activation

To investigate the long-term effect of PTEN and PDK1 deficiency in haematopoietic cells we analysed mice transplanted with PTEN$^{fl/fl}$ Rosa-26cre-ERT cells, PTEN$^{fl/fl}$ PDK1$^{fl/fl}$ Rosa-26cre-ERT and wild type cells eight weeks after cre activation. Our earlier findings showed that engraftment levels of donor cells in mice that received PTEN$^{fl/fl}$ PDK1$^{fl/fl}$ Rosa-26cre-ERT cells drastically dropped four weeks after cre activation compared to loss of PTEN alone (Fig 3.4A). Interestingly, the engraftment level at eight weeks post cre activation was restored and rose to over 60% in the bone marrow (Fig 3.8A) which is similar to wild type transplanted cells but still lower than PTEN transplanted cells. Similarly engraftment of PTEN$^{fl/fl}$ PDK1$^{fl/fl}$ Rosa-26cre-ERT cells was also restored in the spleen and liver (fig 3.8B -C). Mice that received PTEN$^{fl/fl}$ Rosa-26cre-ERT cells maintained a gradual increase in engraftment level over the eight week period post cre activation in the bone marrow, spleen and liver (FIG 3.8 A - C). PTEN and PDK1 deletion was confirmed by PCR using DNA isolated from the bone marrow of recipient mice. We measured the weight of the spleen and liver eight weeks post cre activation and revealed that the mice receiving PTEN$^{fl/fl}$ Rosa-26cre-ERT cells and PTEN$^{fl/fl}$ PDK1$^{fl/fl}$ Rosa-26cre-ERT cells have relatively increased spleen and liver weight compared to mice that received wild type cells (Fig 3.8 E and F).

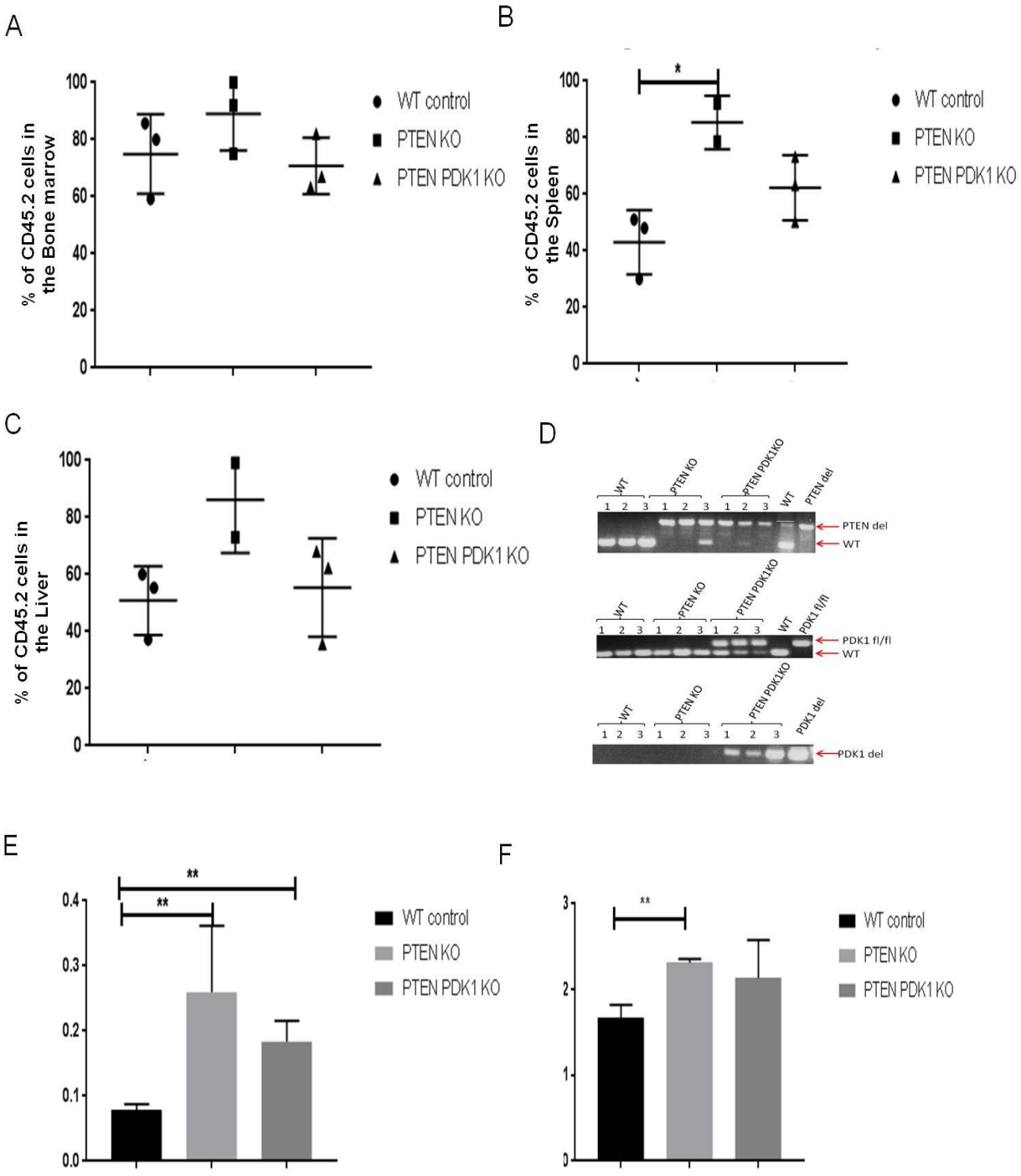


Figure 3.8 Engraftment of PTEN$^{fl/fl}$ Rosa-26cre-ERT and PTEN$^{fl/fl}$PDK1$^{fl/fl}$ Rosa-26cre-ERT cells eight weeks after transplant

(A) Engraftment of donor cells in the bone marrow. (B) Engraftment of donor cells in the spleen (C) Engraftment of donor cells in the liver. (D) PCR confirming deletion of PTEN and PDK1 in the bone marrow (E and F) weight of the spleen and liver respectively.

3.2.8 PTEN deletion induced myeloid and T-cell proliferation

To gain further insight on the long term effect of PTEN and PDK1 deficiency on haematopoietic function, we analysed the composition of the bone marrow to find out if the donor cells were able to maintain the full multi lineage reconstitution eight weeks post cre activation. Mice transplanted with $PTEN^{fl/fl}$ Rosa-26cre-ERT cells and $PTEN^{fl/fl}$ $PDK1^{fl/fl}$ Rosa-26cre-ERT cells showed an increased proliferation of myeloid cells marked by elevated percentage of Mac1-Gr1 double positive cells compared to mice that received wild type cells. Moreover, the B220 population was virtually depleted in mice that received $PTEN^{fl/fl}$ Rosa-26cre-ERT cells and $PTEN^{fl/fl}$ $PDK1^{fl/fl}$ Rosa-26cre-ERT cells. Interestingly, an expansion of CD4 positive cells was observed only in mice that received $PTEN^{fl/fl}$ Rosa-26cre-ERT cells eight weeks Cre activation. A similar proliferation of myeloid lineage cells accompanied with depletion of B220 cells was also observed when PDK1 was deleted in PTEN deficient haematopoietic cells, suggesting that PDK1 has no or only little effect on myeloid proliferation and B-cell depletion. Conversely, the expansion of T-cell lineage cells was only observed in mice that received $PTEN^{fl/fl}$ Rosa-26cre-ERT cells suggesting that PDK1 indeed moderates the effects of loss of PTEN on CD4 T-cells (Fig 3.9A). It is also worth noting that c-Kit population was significantly reduced in mice that received $PTEN^{fl/fl}$ Rosa-26cre-ERT cells compared to mice that received wild type cells (Fig3.9B) suggesting that the LSK population is decreased. Deletion of PDK1 reversed some of the phenotypes caused by PTEN deletion such as reduction of the c-Kit subpopulation and expansion of the CD4 subpopulation. However, it was not able to reverse the expansion of the Mac1/Gr subpopulation and the depletion of B220 subpopulation.

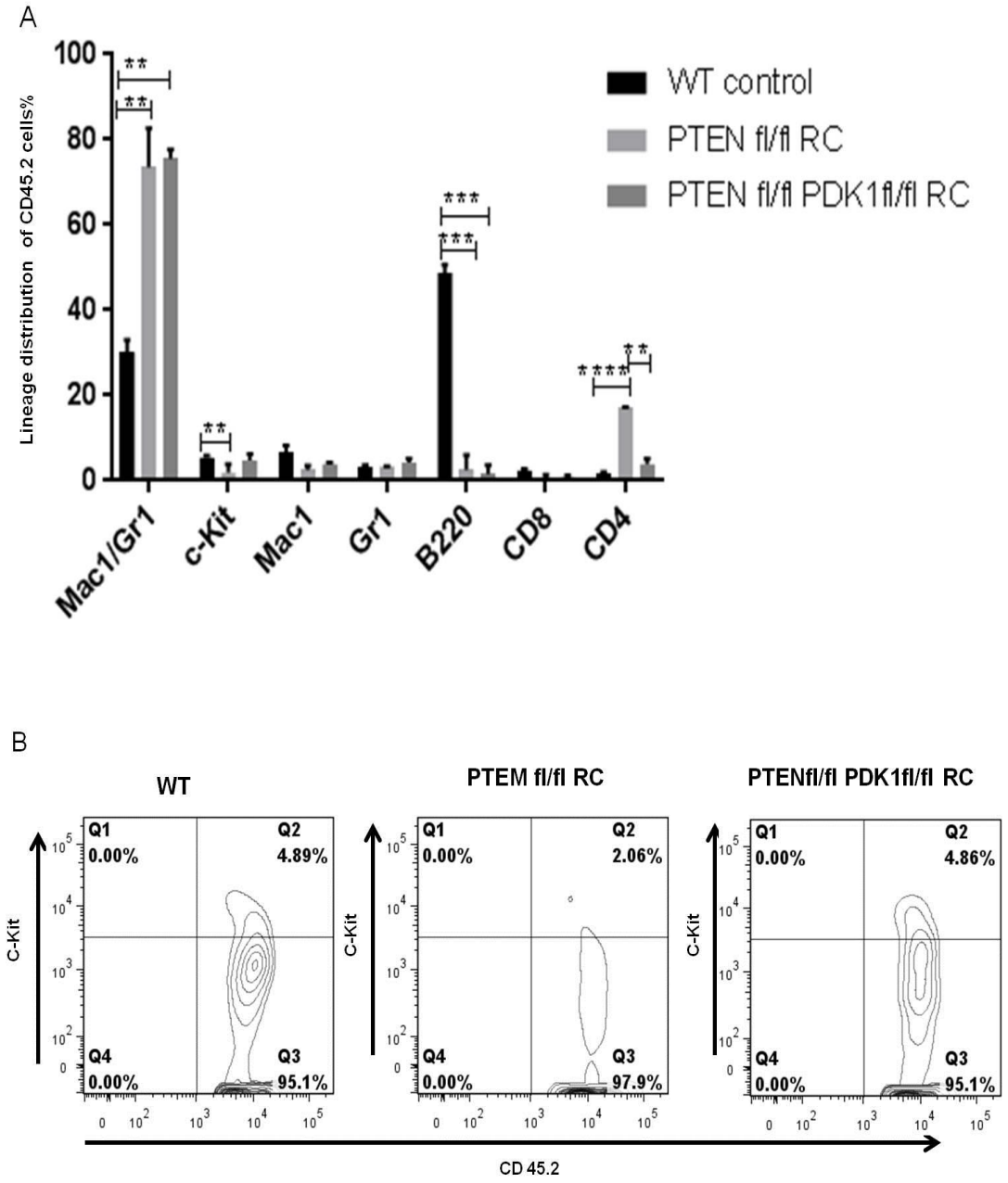


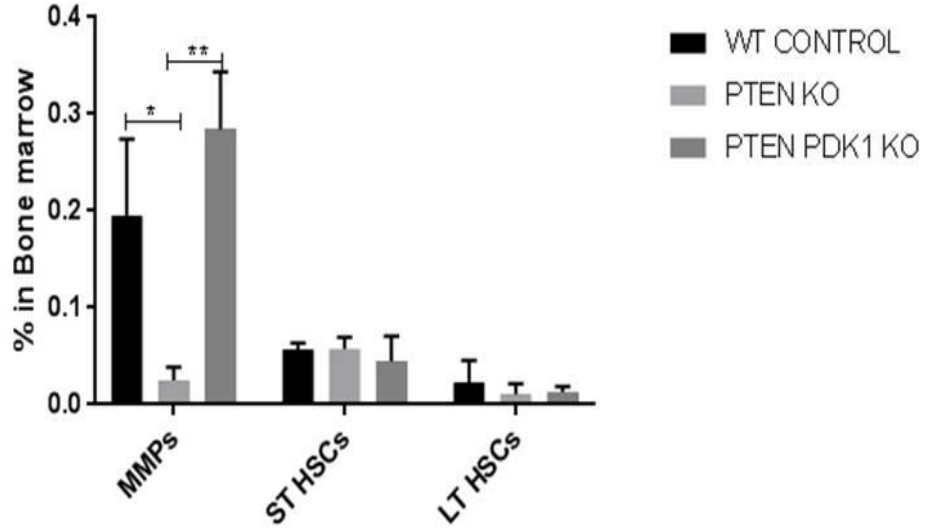
Figure 3.9 Reconstitution with PTEN$^{fl/fl}$ Rosa-26cre-ERT and PTEN$^{fl/fl}$PDK1$^{fl/fl}$ Rosa-26cre-ERT cells 8 weeks post cre activation.

(A) Deletion of PTEN enhanced the proliferation of myeloid cells while virtually depleting the B220 cells whereas deletion of PTEN and PDK1 showed a moderate expansion of myeloid cells and decrease of B220 cells suggesting a mild delay of lineage selection induced by PTEN deletion. (B) Distribution of the c-Kit positive cells within the CD45.2 population showing depletion in mice transplanted with PTEN$^{fl/fl}$ Rosa-26cre-ERT cells.

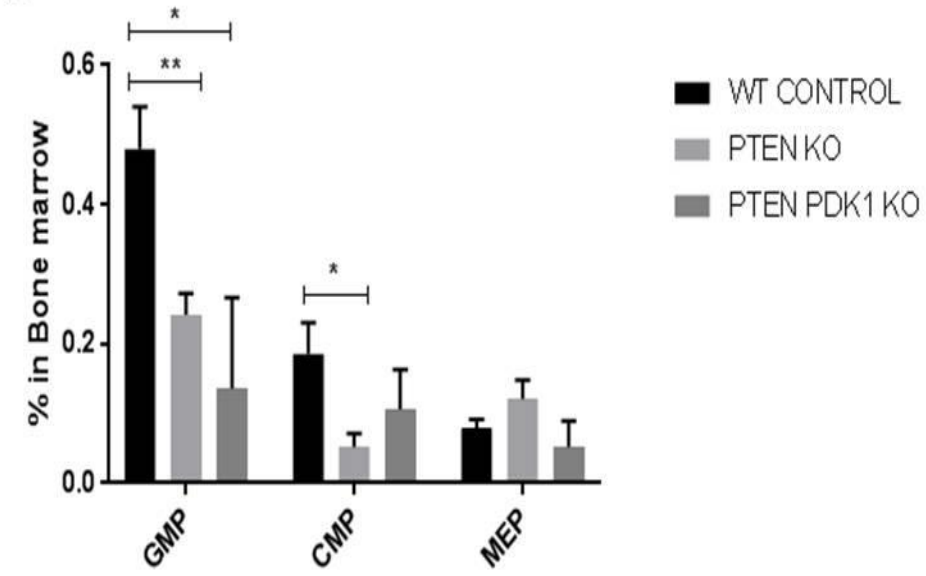
3.2.9 PTEN deletion mediated depletion of HSCs and early progenitors

We have so far demonstrated the effect of PTEN and PDK1 deficiency on lineage reconstitution. To extent this, we investigated the effect of PTEN and PDK1 deficiency on HSCs and early progenitors eight weeks post cre activation. Surprisingly, the MPPs ($Lin^{-/lo}Sca1^{+}c\text{-}Kit^{+}CD150^{-}CD48^{+}$) population was severely depleted in mice that receive $PTEN^{fl/fl}$ Rosa-26cre-ERT cells whereas it was increased in mice that received $PTEN^{fl/fl}$ $PDK1^{fl/fl}$ Rosa-26cre-ERT cells. However, there was no statistically significant different comparing MPPs in mice that received $PTEN^{fl/fl}$ $PDK1^{fl/fl}$ Rosa-26cre-ERT cells to mice transplant with wild type cells (fig 3.10A). We also noticed GMP ($Lin^{-/lo}Sca1^{-}c\text{-}Kit^{+}CD34^{+}CD16/32^{hi}$) population was depleted in mice that received $PTEN^{fl/fl}$ Rosa-26cre-ERT cells and $PTEN^{fl/fl}$ $PDK1^{fl/fl}$ Rosa-26cre-ERT cells whereas the CMP ($Lin^{-/lo}Sca1^{-}c\text{-}Kit^{+}CD34^{+}CD16/32^{lo}$) population was only significantly disturbed in mice that received $PTEN^{fl/fl}$ Rosa-26cre-ERT cells (Fig 3.10B). Together these results suggests deletion of PDK1 can reverse some of the phenotypes caused by PTEN deletion such as depletion of MPP and CMP but could not reverse the depletion of GMPs. Cell cycle analysis of the total bone marrow did not show and significant destruction in mice that received either $PTEN^{fl/fl}$ Rosa-26cre-ERT cells or $PTEN^{fl/fl}$ $PDK1^{fl/fl}$ Rosa-26cre-ERT cells (Fig 3.10C).

A


0.4
0.3
0.2
0.1
0.0
% in Bone marrow
*
**
MMPs
ST HSCs
LT HSCs
WT CONTROL
PTEN KO
PTEN PDK1 KO

B


0.6
0.4
0.2
0.0
% in Bone marrow
*
**
*
GMP
CMP
MEP
WT CONTROL
PTEN KO
PTEN PDK1 KO

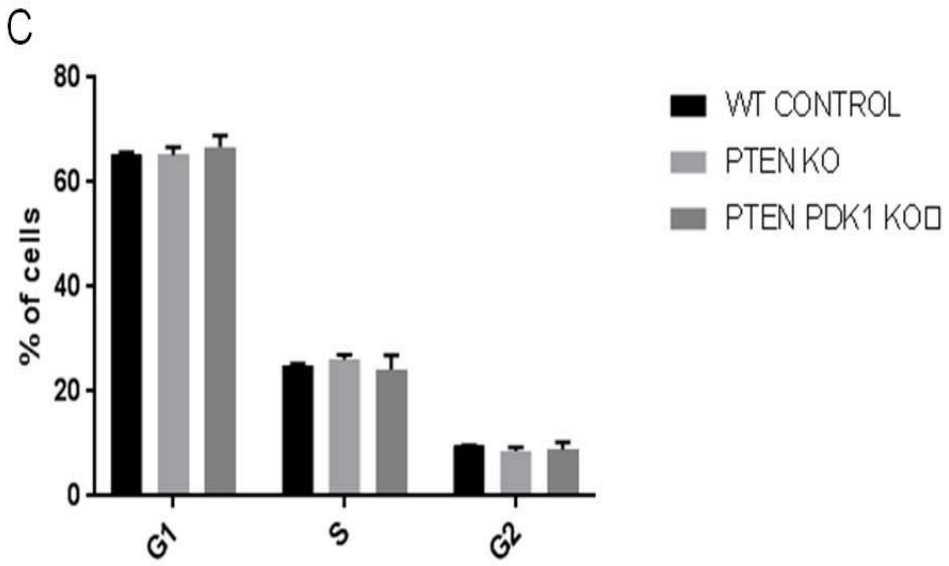


Figure 3.10 Analysis of HSCs and early progenitors 8 weeks after transplant

(A) Distribution of LT-HSCs, ST-HSCs and MPPs four weeks after transplant. (B) Distribution of early progenitors GMP, CMP and MEP four weeks after transplant. (C) Cell circle analysis of total bone marrow cells four weeks after transplant.

3.2.10 Phosphorylation of AKT 308 not required for maintenance of normal haematopoietic cells

We have shown earlier that cells isolated from the bone marrow of mice transplanted with PTEN$^{fl/fl}$Rosa-26cre-ERT have constitutively high levels of phosphorylated AKT at Thr308 and Ser 473 as well as SGK1 Thr256 whereas cells obtained from mice that received PTEN$^{fl/fl}$ PDK1$^{fl/fl}$ Rosa-26cre-ERT cells showed inhibition of AKT Thr308 phosphorylation after cre activation. We again investigated the phosphorylation of AKT Thr308, AKT Ser473 and SGK1 Thr256 eight weeks after activation of cre. Interestingly, phosphorylation of AKT Thr308 was still inhibited eight weeks after cre activation in mice that received PTEN$^{fl/fl}$ PDK1$^{fl/fl}$ Rosa-26cre-ERT cells (Fig 3.11). These results further support our earlier findings that while

activation of AKT Thr308 is dependent on PDK1, it may not be required during normal haematopoiesis.

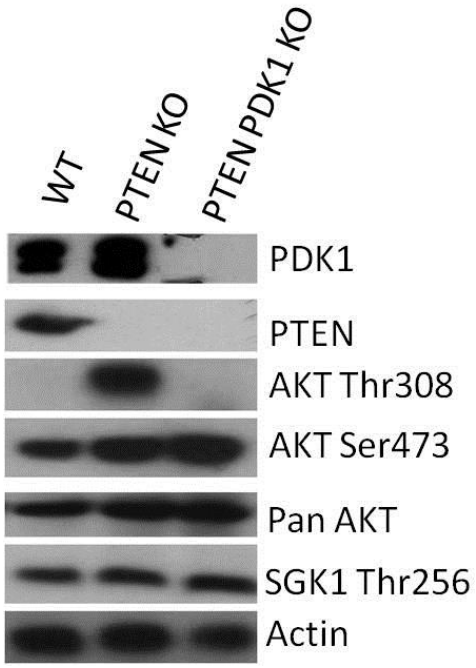


Figure 3.11 Phosphorylation of AKt Thr308 not required for maintain normal haematopoiesis

Western blot analysis of AKT Thr308, Ser473 and SGK1 Thr256 phosphorylation in PTEN$^{fl/fl}$ Rosa-26cre-ERT and PDK1$^{fl/fl}$ PTEN$^{fl/fl}$ Rosa-26cre-ERT bone-marrow cells.

3.3 PTEN deficient HSCs cannot sustain long term reconstitution in irradiated mice

Haematopoietic stem cells are defined by their ability to give rise to all blood cells of multiple lineages when transplanted into irradiated mice. We have demonstrated earlier that PTEN deletion resulted in depletion of early progenitor populations. To functionally confirm the depletion of normal HSCs, we sought to perform transplantation experiments. We first administered tamoxifen (120 µl per day for five days) to induce cre activation in PTEN$^{fl/fl}$ Rosa-26cre-ERT and PTEN$^{fl/fl}$ PDK1$^{fl/fl}$

Rosa-26cre-ERT mice (both CD45.2, n=5). Next we isolated bone marrow cells post treatment and pooled together cells for each genotype. We then sorted HSC ($Lin^{-/lo}Sca1^{+}c\text{-}Kit^{+}CD150^{+}CD48^{-}$), MPP ($Lin^{-/lo}Sca1^{+}c\text{-}Kit^{+}CD150^{-}CD48^{+}$), CMP ($Lin^{-/lo}Sca1^{-}c\text{-}Kit^{+}CD34^{+}CD16/32^{lo}$) and GMP ($Lin^{-/lo}Sca1^{-}c\text{-}Kit^{+}CD34^{+}CD16/32^{hi}$) from the pooled cells. For each genotype we, transplanted $5x10^{2}$ HSCs ($Lin^{-/lo}Sca1^{+}c\text{-}Kit^{+}CD150^{+}CD48^{-}$), $1x10^{4}$ MPPs ($Lin^{-/lo}Sca1^{+}c\text{-}Kit^{+}CD150^{-}CD48^{+}$), $1x10^{4}$ CMPs ($Lin^{-/lo}Sca1^{-}c\text{-}Kit^{+}CD34^{+}CD16/32^{lo}$) and $1x10^{4}$ GMPs ($Lin^{-/lo}Sca1^{-}c\text{-}Kit^{+}CD34^{+}CD16/32^{hi}$) together with $2X10^{5}$ wild type total bone marrow cells (CD45.1) into lethally irradiated mice (CD45.1). Eight weeks after transplantation, we performed a peripheral blood analysis to determine the level of engraftment. We demonstrate that mice transplanted with progenitors from both $PTEN^{fl/fl}$ Rosa-26cre-ERT and $PTEN^{fl/fl}$ $PDK1^{fl/fl}$ Rosa-26cre-ERT lacked engraftment and failed to show any donor reconstitution (Fig 3.12). While mice transplanted with HSCs from $PTEN^{fl/fl}$ Rosa-26cre-ERT showed only a low engraftment level eight weeks after transplant, mice transplanted with $PTEN^{fl/fl}$ $PDK1^{fl/fl}$ Rosa-26cre-ERT HSCs showed good engraftment levels and reconstitution of above 70% of the peripheral blood cells. These results reveal that the reconstitution levels of PTEN deleted HSCs is significantly lower than that of PTEN PDK1 HSCs, strongly suggesting that PTEN HSCs exhaust and that deletion of PDK1 in PTEN deficient HSCs restores normal haematopoietic functions.

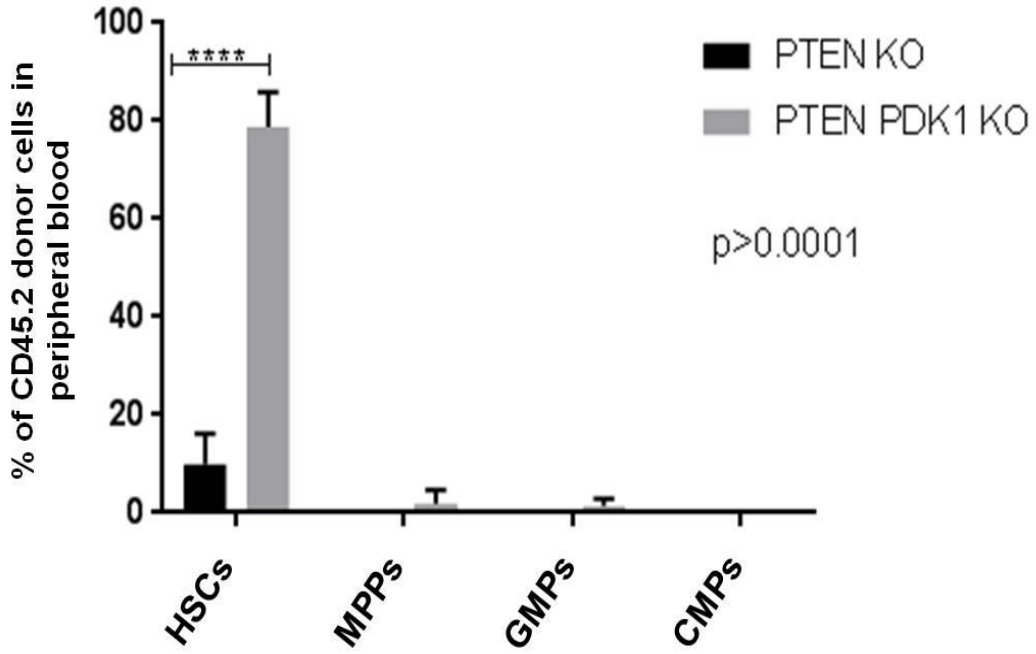


Figure 3.12 PTEN deficient HSCs cannot sustain long term reconstitution in irradiated mice eight weeks after tamoxifen treatment.

For each genotype we, transplanted $5x10^2$ HSCs ($Lin^{-/lo}Sca1^{+}c\text{-}Kit^{+}CD150^{+}CD48^{-}$), $1x10^4$ MPPs ($Lin^{-/lo}Sca1^{+}c\text{-}Kit^{+}CD150^{-}CD48^{+}$), $1x10^4$ CMPs ($Lin^{-/lo}Sca1^{-}c\text{-}Kit^{+}CD34^{+}CD16/32^{lo}$) and $1x10^4$ GMPs ($Lin^{-/lo}Sca1^{-}c\text{-}Kit^{+}CD34^{+}CD16/32^{hi}$) together with $2X10^5$ wild type cells (CD45.1) into lethally irradiated mice (CD45.1) (n=5). Inhibition of PDK1 in PTEN deficient HSCs restored normal haematopoietic function.

Discussion

The haematopoietic system has to be strictly regulated in order to maintain its self-renewal capacity to generate progenitor cells while sustaining a reserve of pluripotent haematopoietic stem cells (Cheung and Mak, 2006). PTEN has been reported to be indispensable in the maintenance of haematopoietic stem cells. A previous study by Yilmaz et al demonstrated that when mice transplanted with $PTEN^{fl/fl}$ Mx-1-Cre were treated with pIpC to activate Cre, the number of PTEN deficient HSCs decreased over time whereas the number of wild type HSCs in the same mice increased (Yilmaz et al., 2006a). Another study by Zhang and colleagues also demonstrated that deleting PTEN in the haematopoietic cells resulted in short term expansion of HSCs but long-term depletion of the HSCs. Moreover, PTEN deficient HSCs could not maintain long term reconstitution in irradiated mice (Zhang et al., 2006). Comparable to previous studies by Yilmaz and Zhang, we used conditional knock out mouse $PTEN^{fl/fl}$ Rosa-26cre-ERT and $PTEN^{fl/fl}$ $PDK1^{fl/fl}$ Rosa-26cre-ERT mice to investigate the role of PTEN and PDK1 in normal haematopoiesis. We transplanted bone marrow cells from $PTEN^{fl/fl}$ Rosa-26cre-ERT and $PTEN^{fl/fl}$ $PDK1^{fl/fl}$ Rosa-26cre-ERT mice (both CD45.2) to lethally irradiated recipient mice (CD45.1) and induce cre activation four weeks later when the mice showed successful engraftment and fully reconstitution with multi-lineage blood cells. Our analysis was only carried out up to eight weeks due early onset of leukaemia in mice that receive $PTEN^{fl/fl}$ Rosa-26cre-ERT cre cells.

Myeloid proliferation as an immediate effect of PTEN deletion has been reported in several studies (Kalaitzidis et al., 2012, Magee et al., 2012, Tesio et al., 2013). Similarly, we demonstrated that deletion of PTEN in the haematopoietic compartment

leads to expansion of double positive Mac1–Gr1 myelocytes while depleting $B220^{+}$ Lymphocytes just four weeks post deletion of PTEN. Furthermore, consistent with previous data from other studies we demonstrate that four weeks and eight weeks post activation of cre, $PTEN^{fl/fl}$ Rosa-26cre-ERT HSCs and early progenitors gradually decrease over time suggesting that the haematopoietic stem cell pool is getting exhausted. Interestingly mice transplanted with $PTEN^{fl/fl}$/$PDK1^{fl/fl}$ cells showed a slower or lesser degree of HSC and early progenitor depletion (Figure 3.10). We also observed increase in the spleen and liver sizes in mice recipient post cre mediation which has also been previously reported as a result of extramedullary haematopoiesis (Tesio et al., 2013). Eight weeks after Cre activation mice that received $PTEN^{fl/fl}$ Rosa-26cre-ERT cre cells showed a much more drastic reduction in MPPs ($Lin^{-/lo}Sca1^{+}c\text{-}Kit^{+}CD150^{-}CD48^{+}$) whereas minimum impact was shown on LT-HSCs ($Lin^{-/lo}Sca1^{+}c\text{-}Kit^{+}CD150^{+}CD48^{-}$) and ST-HSCs ($Lin^{-/lo}Sca1^{+}c\text{-}Kit^{+}CD150^{-}CD48^{-}$). Tesio and colleagues have argued that PTEN deletion has no effect on the number of quiescent and dormant HSCs (Tesio et al., 2013) contradicting the finding that Pten is important regulator of HSC proliferation and self-renewal (Cheung and Mak, 2006, Zhang et al., 2006, Yilmaz et al., 2006b). In addition, we further showed that PTEN deletion lead to depletion of GMPs ($Lin^{-/lo}Sca1^{-}c\text{-}Kit^{+}CD34^{+}CD16/32^{hi}$) and CMPs ($Lin^{-/lo}Sca1^{-}c\text{-}Kit^{+}CD34^{+}CD16/32^{lo}$). Our results are consistent with finding from Zhang and colleagues demonstrating a slight decrease in GMP and CMP population. We demonstrated that in mice transplant with $PTEN^{fl/fl}$ $PDK1^{fl/fl}$ Rosa-26cre-ERT cells in contrast to mice transplant with $PTEN^{fl/fl}$ Rosa-26cre-ERT cells showed comparable MPs and CMP population eight weeks post cre mediation to mice transplanted with wild type cells. In our hands, the ST-HSCs and LT-HSCs did not show a statistical significant difference in control mice compared to mice that received

PTEN$^{fl/fl}$ PDK1$^{fl/fl}$ Rosa-26cre-ERT cells and PTEN$^{fl/fl}$ Rosa-26cre-ERT cells. Together our results suggest that simultaneous deletion of PTEN and PDK1 may have delayed or temporally restored normal haematopoietic function of PTEN depleted HSC. To confirm these claim we demonstrated that HSCs isolated from PTEN$^{fl/fl}$ PDK1$^{fl/fl}$ Rosa-26cre-ERT mice were able to restore engraftment and reconstitute lethally irradiated mice. Consistent with our results, inhibition of mTOR downstream of PDK1 has been shown to restore normal haematopoietic stem cell function (Yilmaz et al., 2006b, Kalaitzidis et al., 2012).

Chapter 4 PTEN and PDK1 in Leukaemogenesis

4.1 A brief introduction

The PI3K pathway has been associated with several roles such as proliferation, survival and differentiation of haematopoietic cells. Consequently, deregulation of this pathway has been associated with development of leukaemia as well as other types of cancer. Thus, enzymes involved in PI3K pathway enzymes are frequently mutated in cancer (Fransecky et al., 2015). Inhibitors targeting key enzymes such as PI3K, AKT and mTOR are being developed for the treatment of several cancers such as colorectal cancer (Zhang et al., 2011) and in some cases are endorsed as the standard of care. Abnormal activation of AKT and mTOR have long been recognized as key downstream effects of mutated PI3K pathway resulting in growth advantage of AML stem cells (Vanhaesebroeck et al., 2012).

PTEN is a key regulator of the PI3K pathway and loss of function of PTEN results in increased activation of PDK1 and AKT. PDK1 directly activate Akt and mTOR complex 1, both of which have been directly linked to leukaemogenesis. Moreover, PTEN deletions or mutation have been reported in about 5% to 10% of T-ALL cases (Gutierrez et al., 2009) and less than 2% of AML cases (Zeisig et al., 2012) . Most studies investigating the PI3K pathway and its relation to cancer used approaches such as targeting components of the PI3K pathway using inhibitor or gene silencing. While many studies focused on only a single component of the PI3K pathway, I decided to investigate the role of PTEN and PDK1 in leukaemia using conditionally knock-out mouse models were either PTEN, PDK1 or both simultaneously were deleted in the well-defined haematopoietic stem/progenitor cells. Using these mouse models, I can

compare the impact of loss of PTEN, PDK1 or both on leukaemogenesis. Given the important role of PDK1 in activating the various components downstream of the PI3K pathway I further aimed to elucidate the function of PDK1 by using additional specific knock-in (KI) mice (PDK1 L155E MG and PDK1 K465E). In these models, PDK1 L155 mice have disrupted PDK1-PIF pocket domain whereas the PDK1 K465 have a defective PDK1-PH domain. These two domains are critical for the distinctive function of PDK1 i.e. phosphorylation of AKT Thr308 via the PH domain and phosphorylation of SGK1, RSK, S6K and PKC via the PIF pocket.

4.2 Role of PTEN and PDK1 in peripheral blood engraftment and lineage contribution

To study the effect of PTEN and PDK1 deletion alone or in combination in haematopoietic cells, we performed bone marrow transplantation experiments using total bone marrow cells (CD45.2) isolated from $PTEN^{fl/fl}$ Rosa-26cre-ERT, $PDK1^{fl/fl}$ Rosa-26cre-ERT, $PTEN^{fl/fl}PDK1^{fl/fl}$ Rosa-26cre-ERT, $PDK1^{L155E/L155E}PTEN^{fl/fl}$ Rosa-26cre-ERT, $PDK1^{MGK465/MGK465}PTEN^{fl/fl}$ Rosa-26cre-ERT and $PDK1^{MGK465/MGK465}$ Rosa-26cre-ERT. In order to distinguish donor from competitor cells upon transplantation, our transgenic mice (donor) were C57BL/6J (CD45.2) whereas the recipient mice were wild type C57BL/6SJL (CD45.1). We transplanted 1 mio donor cells (CD45.2) together with 0.2 mio rescue cells (CD45.1) into lethally irradiated SJL mice (CD45.1). To determine the ability of the different donor cells to engraft, we carried out FACS analysis on peripheral blood samples taken four weeks after transplantation. Using a panel of antibodies specifically against myeloid markers (Mac1, Gr1), stem and progenitor cell marker (c-Kit), lymphoid markers (CD4, CD8

and B220), as well as CD45.2 and CD45.1 allowed us to determine not only the level of engraftment but also the lineage distribution of haematopoietic cells originating from the donor.

4.2.1. PTEN deletion has no effect on engraftment

As a control, we demonstrated that normal haematopoietic cells harvested from a wild type C57BL6 CD45.2 mouse had the ability to engraft in lethally irradiated recipient mice (Fig 4.1A). The percentage of C57BL6 CD45.2 (donor cells) was determined to be 70% in the peripheral blood of recipient mice four weeks post-transplant. Analysis of peripheral blood in mice transplant with $PTEN^{fl/fl}$Rosa-26cre-ERT cells also revealed engraftment levels around 70% four weeks after transplant comparable to the observation with wild type cells (Fig4.1B). To study effect of PTEN deletion in haematopoietic cells, we analysed the blood again at four weeks post-treatment with tamoxifen. Genotyping PCR on genomic DNA isolated from peripheral blood cells revealed a complete deletion of the PTEN floxed allele four weeks after tamoxifen treatment (Fig 4.1C). Further analysis of peripheral blood was carried out again at eight weeks and twelve weeks after tamoxifen administration. As expected, the percentage of donor cells further increased to around 75% and 85% at 4 weeks and 8 weeks respectively and remained at this level at 12 weeks, which was very similar to the observations of the wild type cells.

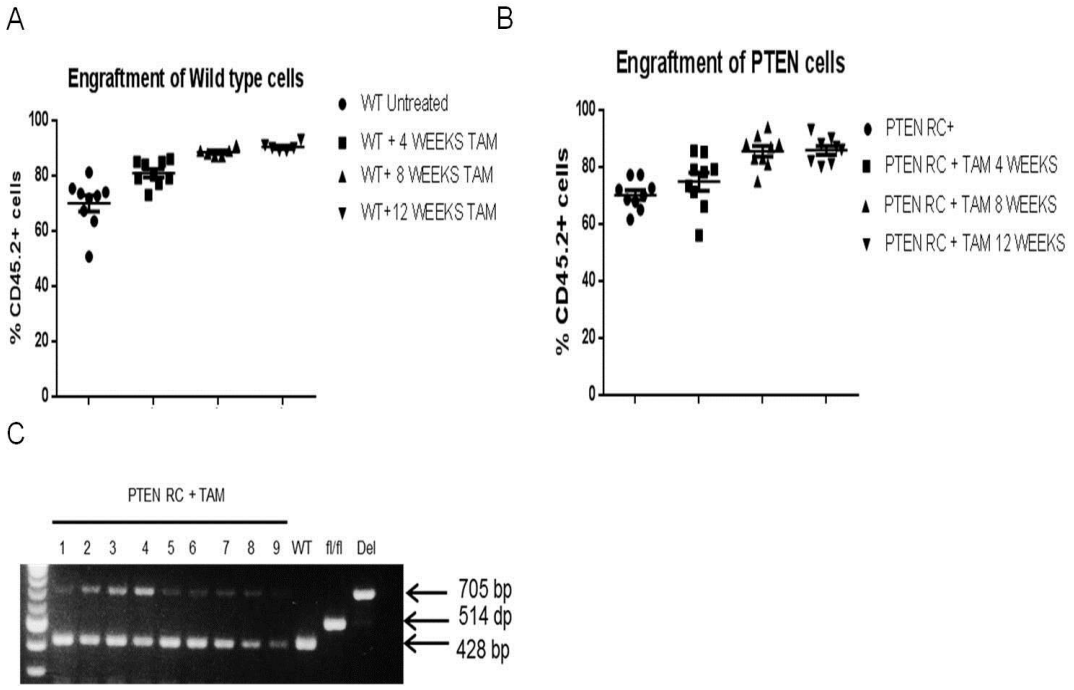


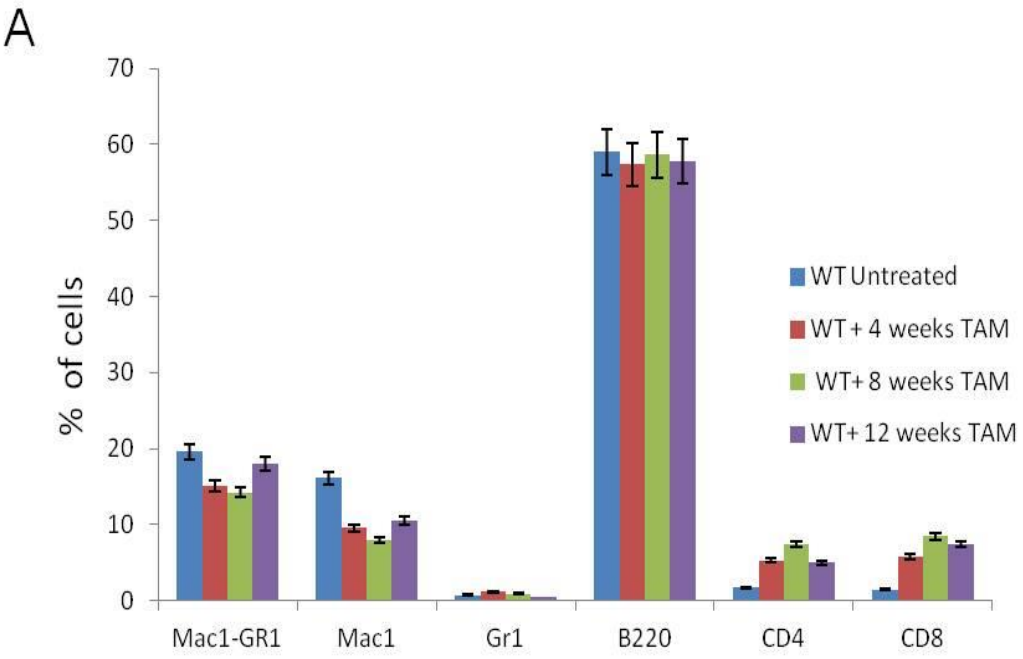
Figure 4.1 Engraftment of wild type and PTEN$^{fl/fl}$ Rosa-26cre-ERT cells

Total bone marrow from wild type C57BL6 mouse and PTEN$^{fl/fl}$ Rosa-26cre-ERT mouse (both CD45.2) (1 mio cells) was transplanted into SJL (CD 45.1) recipient mice (n=10) together with 0.2 mio CD45.1 wild type bone marrow cells for rescue. (A) Percentage of wild type donor cells before and after tamoxifen treatment. (B)Percentage of PTEN$^{fl/fl}$ Rosa-26cre-ERT donor cells before and after tamoxifen treatment. (C) PCR analysis of blood 4 weeks after tamoxifen treatment (1 to 9 represent each mouse transplanted with PTEN$^{fl/fl}$ Rosa-26cre-ERT after cre activation). PCR controls are indicated.

4.2.2 PTEN deletion has influence on lineage selection

In addition to determining the percentage of engraftment, we also monitored the lineage distribution of haematopoietic cells derived from the donor before and after cre activation. We further demonstrate that normal haematopoietic cells did not only engraft but also lead to multi-lineage reconstitution of the blood (Fig 4.2A). Although the normal cells showed great potential to proliferate as indicated by their increase over the twelve weeks period, the cell population distribution remained unchanged before and after treatment with tamoxifen. In contrast to the control (wild-type cells) transplanted cohort, PTEN deletion resulted in an increase of Mac1 positive myeloid

cells at 8 and 12 weeks as well as Mac1/Gr1 double positive myeloid cell at 12 weeks after tamoxifen treatment while the proportion of Gr1 positive cells remained unchanged (Fig 4.2B). On the other hand, a gradual decrease in B220 B-cells was observed from 4 weeks to 12 weeks after tamoxifen treatment, whereas no significant changes were observed in CD4 and CD8 T-cells. Together this data indicate that PTEN deletion causes hyper-proliferation of myeloid cells while depleting B220 positive lymphoid cells.



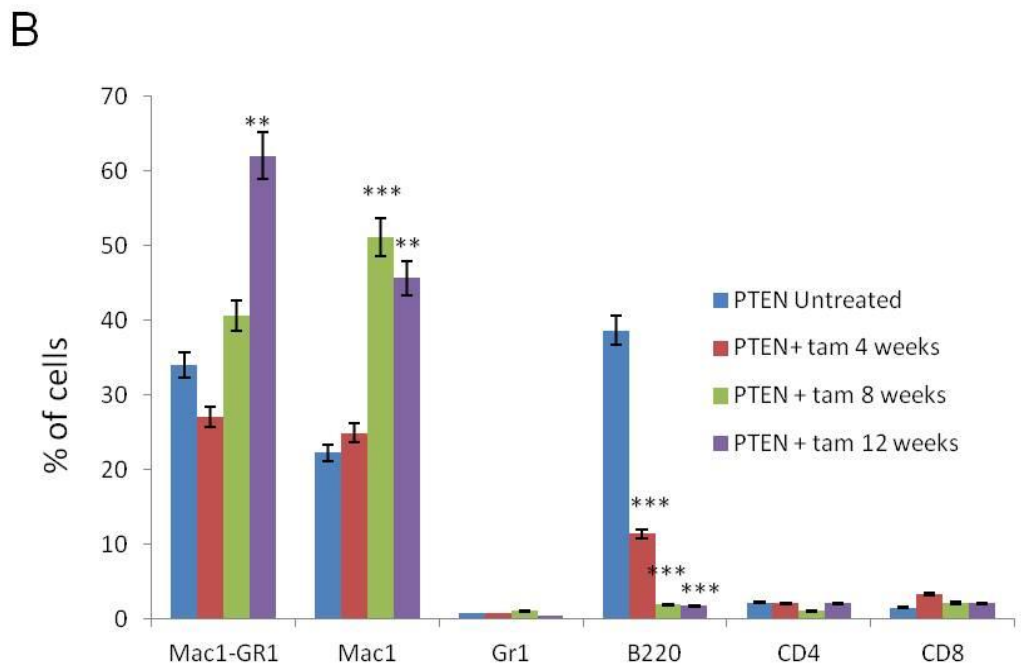


Figure 4.2 PTEN deletion induces lineage selection

A) Immunophenotying of wild type donor cells before and after tamoxifen treatment is shown. (B) Immunophenotying of PTEN$^{fl/fl}$Rosa-26cre-ERT donor cells before and after tamoxifen treatment is shown. Significance (* P <0.05 ** P >0.005 *** P > 0.001)

4.2.3 Deletion of PDK1 impairs the engraftment

PDK1 contributes to cell growth due to its ability to activate a series of signal transducers that are involved in promoting cell proliferation and survival (Bone and Welham, 2007). Consequently, it was anticipated that deletion of PDK1 has a negative effect on cell proliferation and survival. We demonstrate that the level of engraftment of PDK1$^{fl/fl}$Rosa-26cre-ERT cells as determined by the percentage of CD45.2 donor cells in the blood was around 60% at 4 weeks after transplantation, which was comparable to the wild type control. However, the percentage of PDK1$^{fl/fl}$Rosa-26cre-ERT cells dropped to just 2% 4 weeks after tamoxifen treatment (Fig 4.3A). Due to the depleted number of cells after cre activation, I could not confirm the *in vivo*

deletion of PDK1 in this experimental set up. At eight weeks and twelve weeks the percentage of donor cells further dropped to almost 0%. These results suggest that PDK1 is essential for cell growth and proliferation as suggested by other studies (Venigalla et al., 2013, Park et al., 2013).

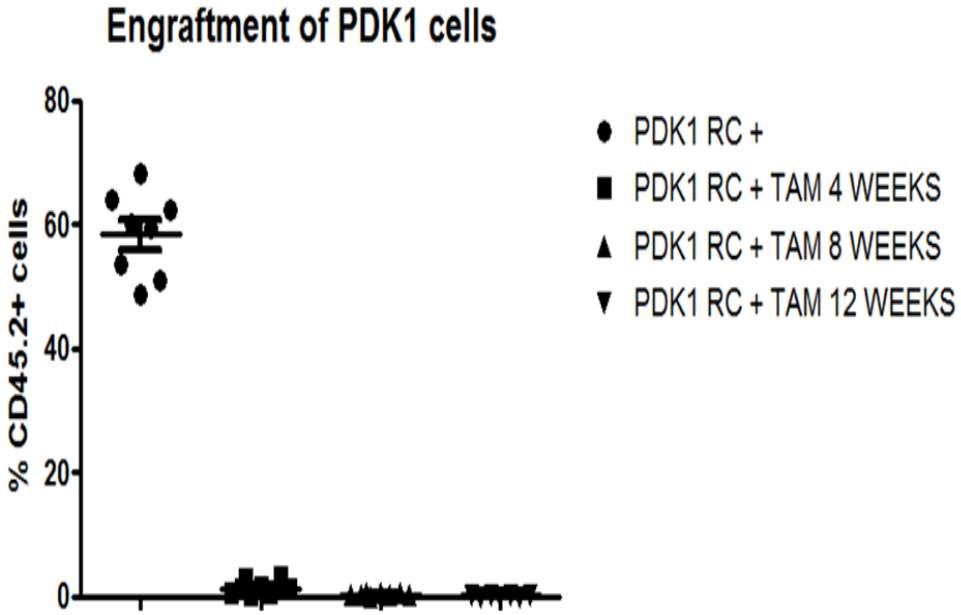


Figure 4.3 Reconstitution of recipient mice with PDK1$^{fl/fl}$ Rosa-26cre-ERT cells

Total bone marrow from type PDK1$^{fl/fl}$ Rosa-26cre-ERT mouse (CD45.2) (1 mio cells) was transplanted into SJL (CD 45.1) recipient mice (n=10), 0.2 mio CD45.1 bone marrow cells used for rescue. % of wild type (CD45.2) before and after tamoxifen treatment.

4.2.4 PDK1 deletion impairs the engraftment levels in PTEN deleted cells

PTEN$^{fl/fl}$PDK1$^{fl/fl}$Rosa-26cre-ERT cells showed comparable engraftment levels of about 70% four weeks after transplantation. Interestingly, the engraftment levels dropped to around 50% four weeks after treament with tamoxifen (Fig 4.4A) and remained at this lower level throughout the course of the experiment. This result is in stark contrast to the enhanced engraftment observed in PTEN deleted cells and the loss of engraftment observed in PDK1 deleted cells, suggesting that deletion of PTEN

rescues PDK1 KO engraftment phenotypes. Genotyping PCR of genomic DNA isolated from peripheral blood demonstrated complete deletion of PTEN (Fig 4.4B). The deletion of PDK1 alleles was to some degree achieved as shown with the apperence of the deleted band (Fig 4.4C). However the presence of a floxed band was unexpected and therefore it was inconclusive to confim a complete deletion by PCR alone. In order to demonstrate a complete loss of PDK1 protein in the compound KO mice, we also performed a western blot using cells isolated form bone marrow, which showed that completed deletion of PTEN and PDK1 was achieved (Fig 4.4D).

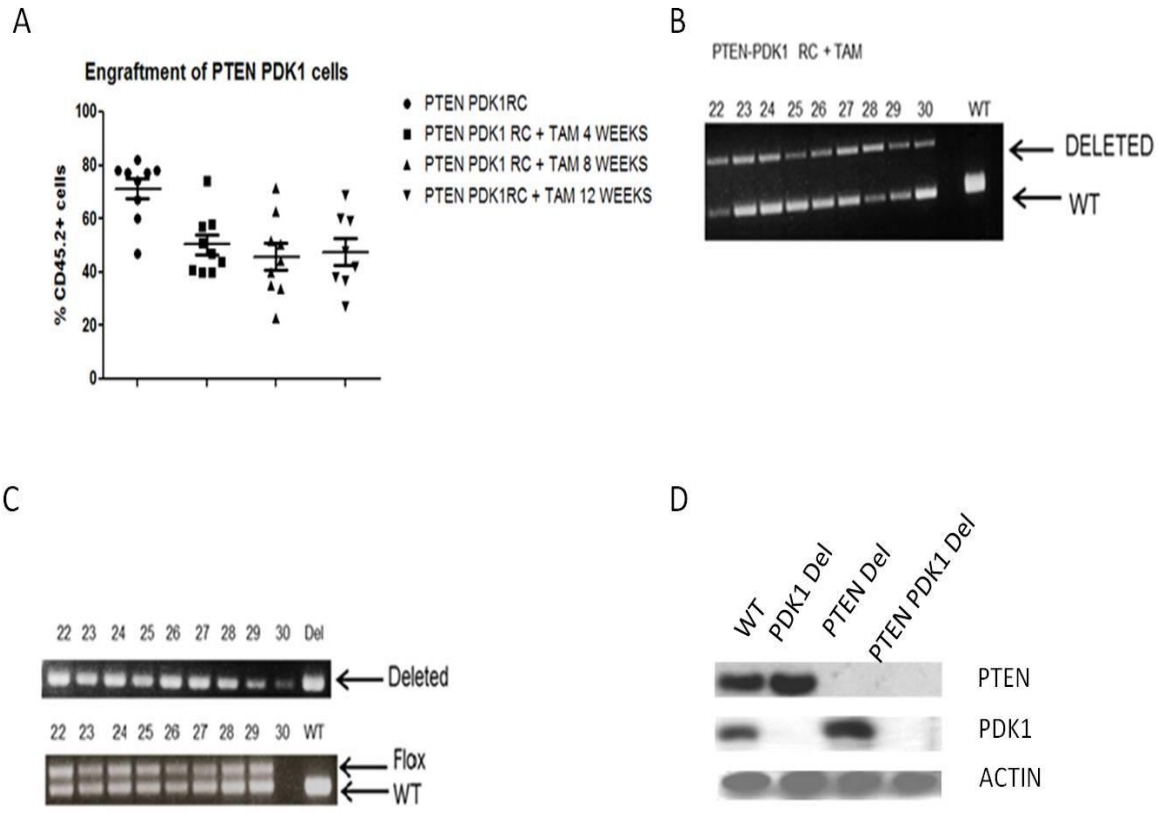


Figure 4.4 Engraftment of PTEN $^{fl/fl}$PDK1$^{fl/fl}$ Rosa-26cre-ERT cells

Total bone marrow cells from PTEN$^{fl/fl}$PDK1$^{fl/fl}$ Rosa-26cre-ERT mouse (1 mio cells CD45.2 cells) transplanted lethally irradiated SJL mice (CD45.1 n=10) (0.2 mio CD45.1 cells used for rescue). (A) % of CD45.2 Donor cells 4 weeks after transplantation and every 4 weeks after tamoxifen treatment. (B) PCR analysis of blood 4 weeks after tamoxifen treatment showing complete deletion of PTEN.PCR controls are indicated. (C) PCR analysis of PDK1 allele 4 weeks after tamoxifen. PDK1 deletion confirmed by presence of PDK1 null band. 22 to 30 and WT represent samples from each experimental mouse. (D) Western blot showing complete deletion of PTEN and PDK1 in PTEN$^{fl/fl}$ PDK1$^{fl/fl}$ Rosa-26cre-ERT cells after cre activation.

4.2.5 Simultaneously deleting PTEN and PDK1 influences lineage distribution of haematopoietic cells in recipient mice

To determine the effect of simultaneous deletion of PTEN and PDK1 on the distribution of myeloid and lymphoid cells, we analysed the distribution of PTEN/PDK1 deleted cells in the blood. Surprisingly we found that the population of myeloid cells was increased after tamoxifen treatment whereas the population of B cells was reduced (Fig 4.5), a phenotype close to that of mice with PTEN deleted cells. However, a closer look at the populations involved and the dynamic of the changes revealed significant differences between loss of PTEN and loss of both PTEN and PDK1. In the experiment with PTEN deletion, Mac-1 positive cells were increased by week 8 and 12 and an increase of Mac1/Gr1 positive cells was only observed at week 12. Interestingly, while both populations were present at similar levels in the blood, no changes were observed for CD4 or CD8 T-cells. In contrast, PTEN/PDK1 deletion led to increased Mac1/Gr1 cells in week 4, 8 and 12 and only minimal increase of Mac1 positive cells by week 12. Strikingly, more than twice as many Mac1/Gr1 cells compared to Mac1 positive cells are present in the blood. Moreover, increases in the CD4 and CD8 T-cell populations are present in weeks 4 and 8 and week 4, respectively, suggesting that loss of PDK1 in the PTEN deleted background influences not only the engraftment properties but also the lineage distribution of haematopoietic cells in recipient mice. Although the PTEN/PDK1 KO mice grossly follow PTEN KO phenotype, loss of PDK1 also has its impact on lineage distribution.

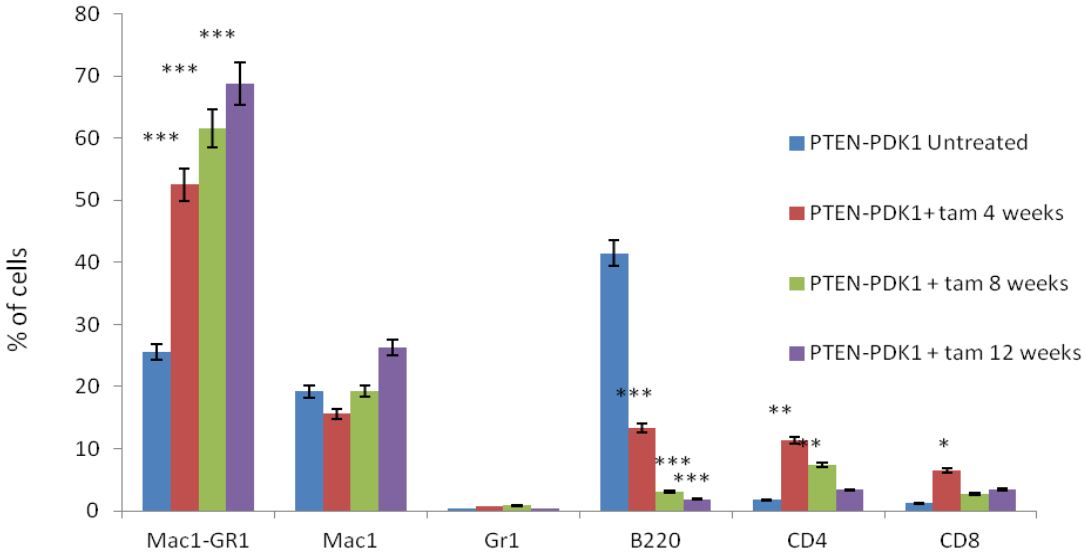


Figure 4.5 Reconstirution with PTEN $^{fl/fl}$ PDK1 $^{fl/fl}$ Rosa-26cre-ERT cells

Immunophenotying of PTEN PDK1 cells before and after tamoxifen treatment is shown. Significance (* P <0.05 ** P >0.005 *** P > 0.001)

4.3 Roles of PTEN and PDK1 in leukaemogenesis

It has been shown before that PTEN deletion in haematopoietic cells lead to leukaemia (Guo et al., 2011, Kalaitzidis et al., 2012, Magee et al., 2012, Yilmaz et al., 2006b, Zhang et al., 2006). The strength of our experimental model is that we can genetically knockout PDK1 in PTEN deleted cells and therefore investigate the role of PDK1 in PTEN null leukaemia. While mice tranplanted with PDK1$^{fl/fl}$Rosa-26cre-ERT or WT cells remained leukaemia free, all mice transplanted with PTEN$^{fl/fl}$Rosa-26cre-ERT cells where the deletion of PTEN had been induced in haematopoietic cells succumbed to leukaemia with a latency between 50 days and 140 days after PTEN deletion (Fig 4.6). Remarkably, mice transplanted with PDK1$^{fl/fl}$PTEN$^{fl/fl}$Rosa-26cre-ERT showed no signs of disease until around 160 days after tamoxifen treatment. More importantly, the disease that was oberved from day 160 in these animals showed a different kinetics

and indeed 40% of the mice remained disease free beyond 400 days. Taken together, our data demonstrate that inhibition of PDK1 significantly delays the onset of PTEN mediated leukaemia and suggest that PDK1 is a critical mediator in the loss of PTEN mediated leukaemogenesis. This data is consistant with data from previous studies showing that PDK1 has antagonistic effect to PTEN and therefore could potentialy offset the effects of loss of PTEN function (Iwanami et al., 2009). However, the simultanious deletion of PDK1 and PTEN did not completely reverse the leukaemogenesis suggesting that some unknown mechanism might be triggered in the mice that came down with leukaemia to compensate for loss of PDK1.

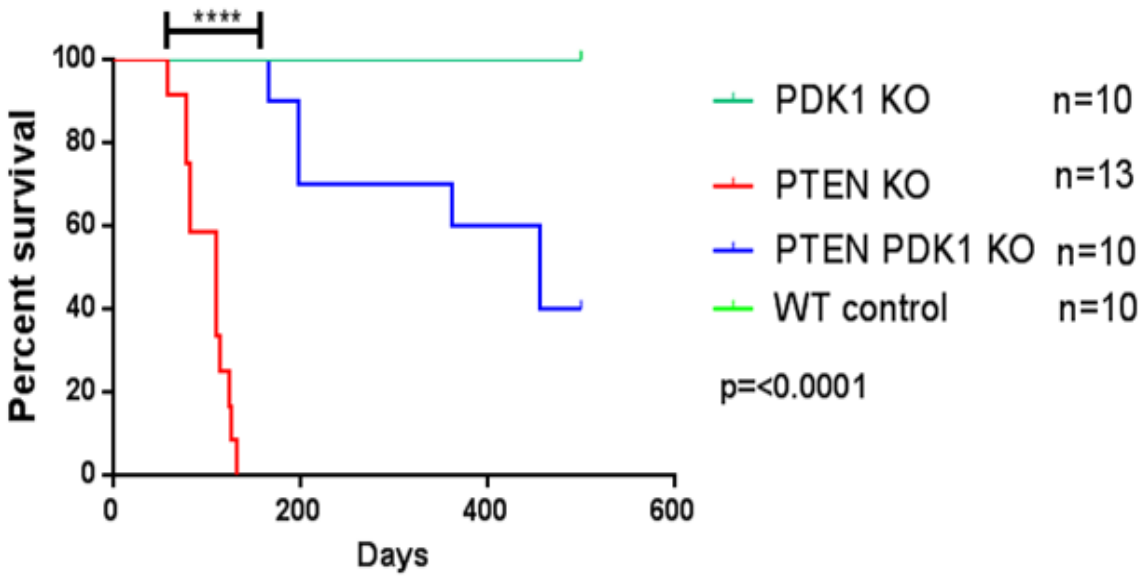


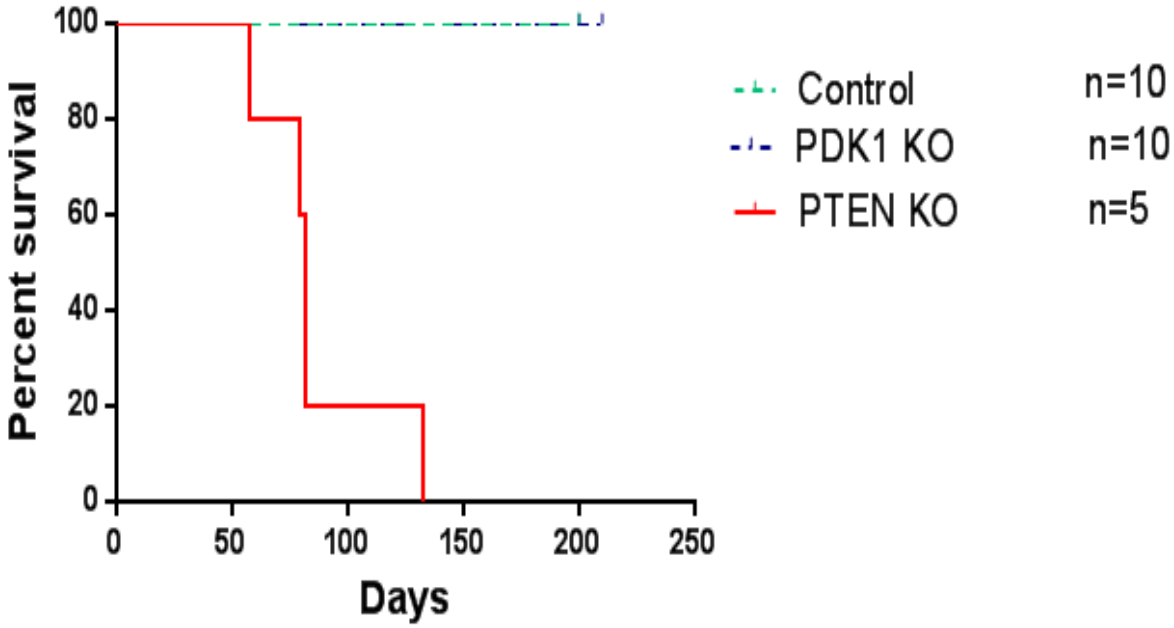
Figure 4.6 PTEN deletion induced leukaemogenesis

Graph showing the development of leukaemia in mice transplanted with PTEN$^{fl/fl}$ Rosa-26cre-ERT cells compared to mice transplanted with PDK1$^{fl/fl}$ PTEN$^{fl/fl}$ Rosa-26cre-ERT cells and mice transplanted with wild type cells (P value >0.0001 PTEN$^{fl/fl}$ Rosa-26cre-ERT vs PDK1$^{fl/fl}$ PTEN$^{fl/fl}$ Rosa-26cre-ERT).

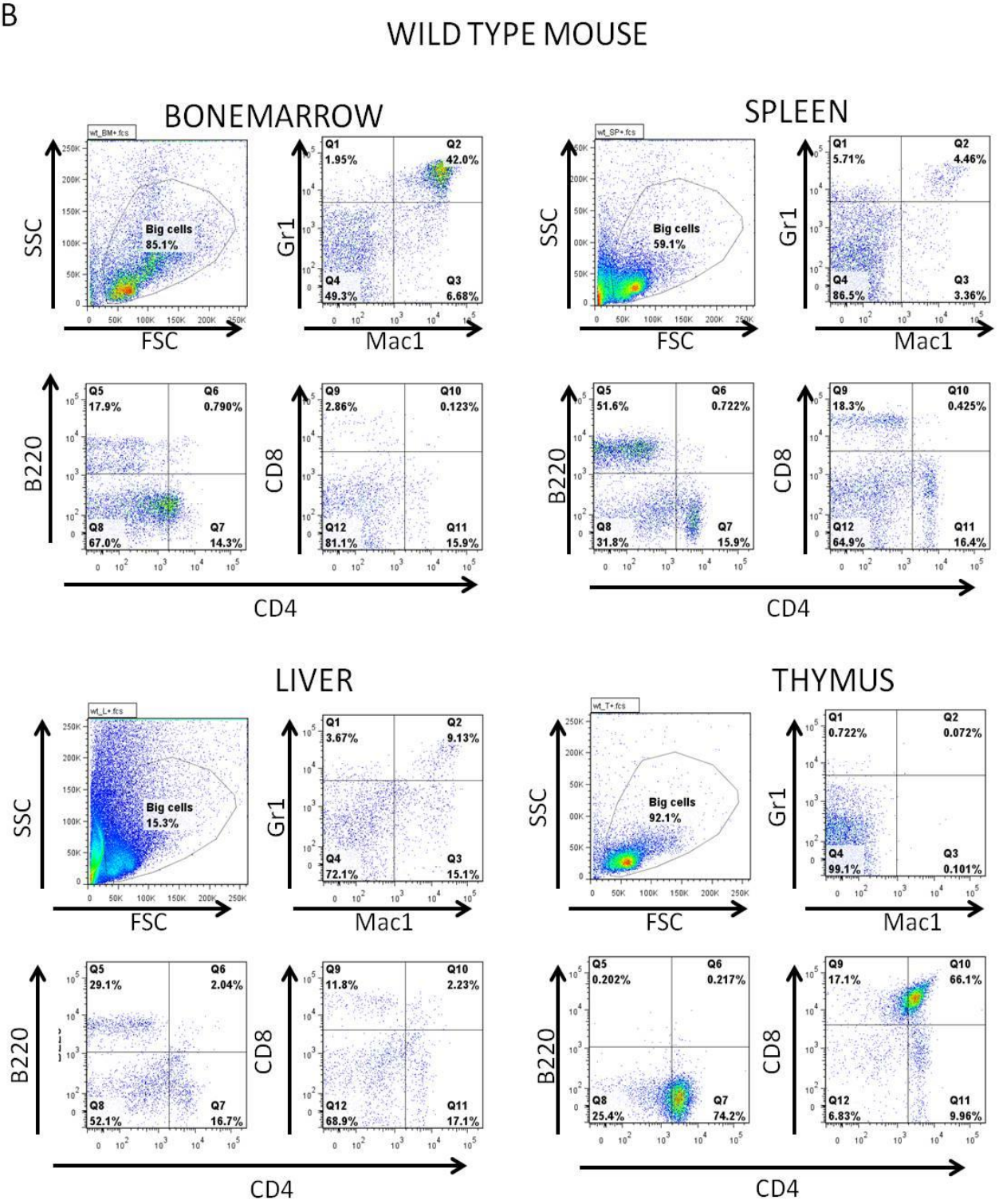
4.3.1. PTEN deletion in adult haematopoietic cells results in ALL and AML

Our previous data from the analysis of blood samples showed that PTEN deleted cells were able to produce a short term multi-lineage reconstitution in peripheral blood before eventually turning into myeloid proliferation while depleting B cells. We previously showed that approximately 50 days after tamoxifen treatment, the mice that received $PTEN^{fl/fl}$Rosa-26cre-ERT cells started to develop leukaemia and all thirteen mice died by day 140. Bone marrow, spleen, liver and thymus of the all the mice that succumbed to leukaemia were examined. FACS analysis using antibodies against a panel of surface markers including Mac1, Gr1, B220, CD4 and CD8 showed that these mice developed AML, ALL or biphenotypic leukaemia. Our results are comparable to findings from previous studies that also reported the AML and ALL phenotypes in PTEN mediated leukaemogenesis (Yilmaz, 2006). The normal phenotype of a wild type mouse showed around 60% myeloid cells and 40% lymphoid cell in the bone marrow (Fig 4.7B). The distribution of haematopoietic cells in the spleen reveals around 17% are myeloid cells and 83% are lymphocytes with majority of cells belonging to B-cell lineage. The distribution of haematopoietic cells in the liver showed to be around 30% myeloid and 70% lymphoid cells while the thymus showed to compose of only T lymphocytes. Five out of thirteen mice transplanted $PTEN^{fl/fl}$ Rosa26 cre cells came down with AML (Fig 4.7A). FACS analysis of the bone marrow of AML mice showed almost a complete infiltration of the bone marrow with myeloid cells and in most cases more than 70% the cells were positive for both Mac1 and Gr 1 suggesting an shift in the turnover of myeloid cells (Fig 4.7C). The spleen and liver of AML mice also showed increased proportion of myeloid cells with a

majority of the cells again positive for both Mac1 and Gr1. The proportion of lymphocytes was shown to be greatly reduced in the bone marrow, spleen, and liver. Myeloid cells were not present in the thymus in any of the mice that came down with AML.



B


WILD TYPE MOUSE
BONEMARROW
wt_BM+.fcs
SSC
FSC
Big cells
85.1%
Gr1
Mac1
Q1
1.95%
Q2
42.0%
Q4
49.3%
Q3
6.68%
B220
CD4
Q5
17.9%
Q6
0.790%
Q8
67.0%
Q7
14.3%
CD8
Q9
2.86%
Q10
0.123%
Q12
81.1%
Q11
15.9%
SPLEEN
wt_SP+.fcs
Big cells
59.1%
Q1
5.71%
Q2
4.46%
Q4
86.5%
Q3
3.36%
Q5
51.6%
Q6
0.722%
Q8
31.8%
Q7
15.9%
Q9
18.3%
Q10
0.425%
Q12
64.9%
Q11
16.4%
LIVER
wt_L+.fcs
Big cells
15.3%
Q1
3.67%
Q2
9.13%
Q4
72.1%
Q3
15.1%
Q5
29.1%
Q6
2.04%
Q8
52.1%
Q7
16.7%
Q9
11.8%
Q10
2.23%
Q12
68.9%
Q11
17.1%
THYMUS
wt_T+.fcs
Big cells
92.1%
Q1
0.722%
Q2
0.072%
Q4
99.1%
Q3
0.101%
Q5
0.202%
Q6
0.217%
Q8
25.4%
Q7
74.2%
Q9
17.1%
Q10
66.1%
Q12
6.83%
Q11
9.96%

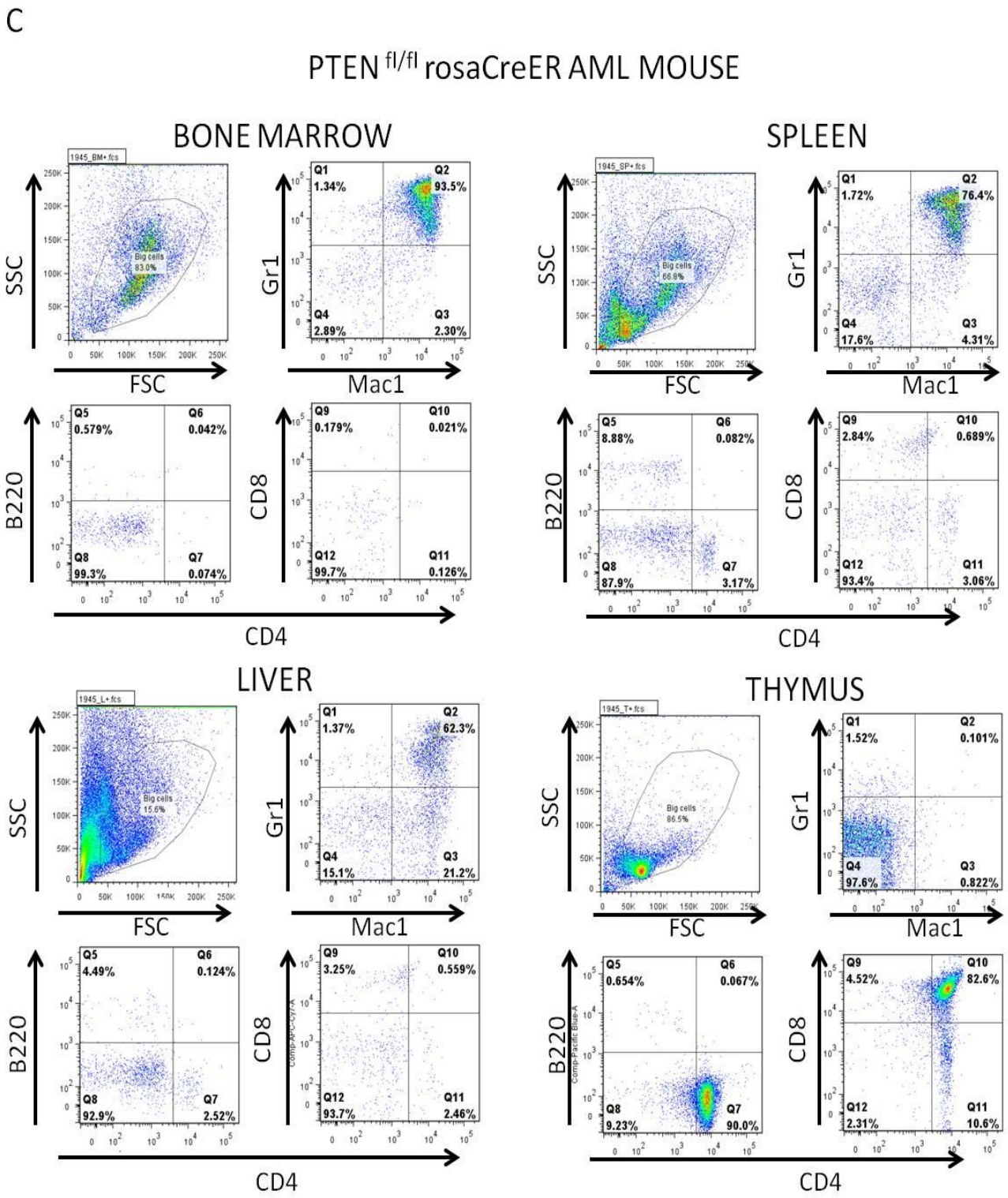
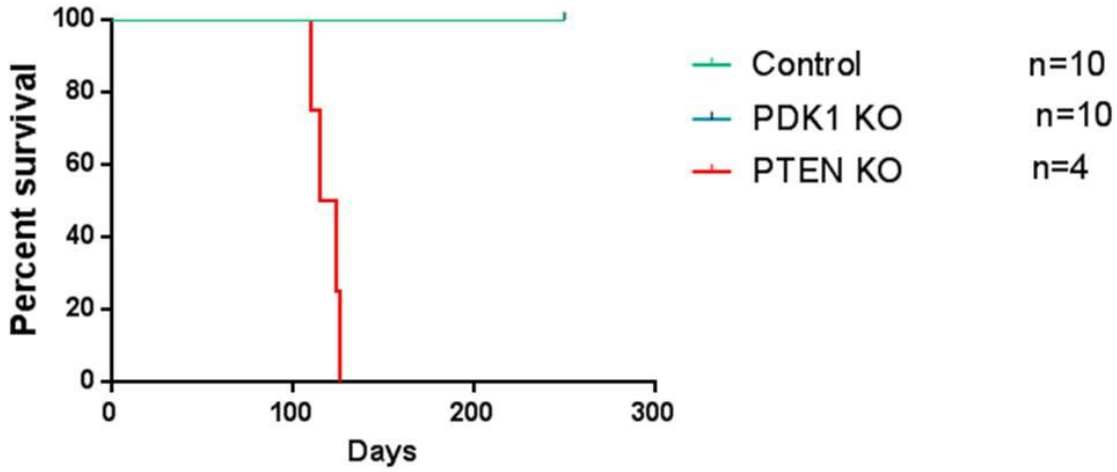


Figure 4.7 PTEN deletion induced AML

(A) Graph showing survival of mice after PTEN deletion. Five out of thirteen mice came down with AML. (B) FACS profile of a wild type mouse. (C) FACS profile of AML mouse.

Four out of thirteen mice transplanted with PTEN$^{fl/fl}$ Rosa-26cre-ERT cells came down with ALL. All four mice came down with ALL between 100 and 140 days (Fig 4.8A). Mice that developed ALL had a very large thymus while the spleen and liver in some of the mice appeared large the majority of ALL mice had normal size liver and spleen. Examination of the bone marrow of a typical ALL mice showed an infiltration of T-cells into the bone marrow making over 50% of the total bone marrow. A typical ALL mouse presented with majority of the tumour cells double positive for CD4 and CD8 (CD4^{+} CD8^{+}) (Fig 4.8B). The spleen and the liver of ALL mice also showed almost a complete infiltration of these organs by T lymphocyte.

A



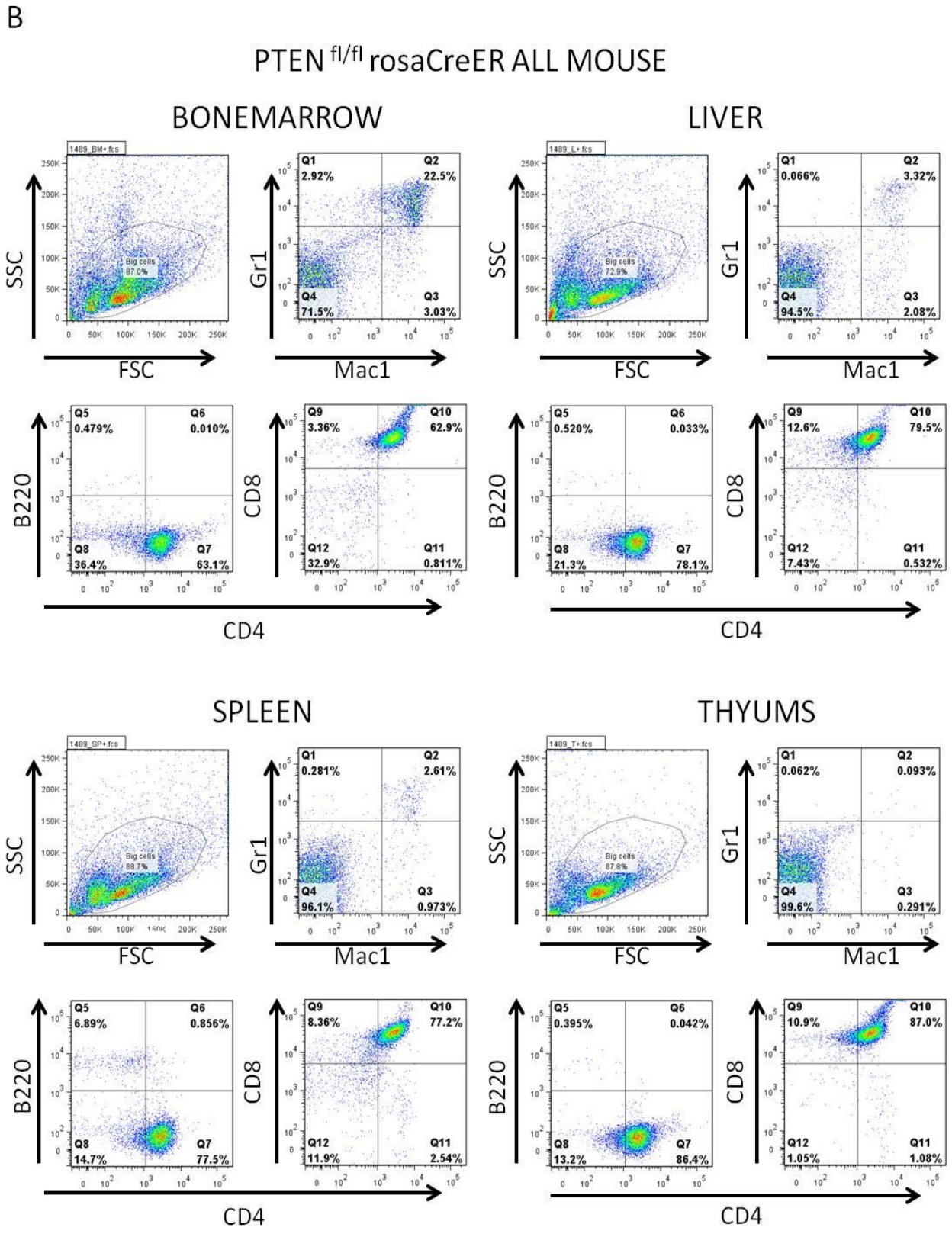
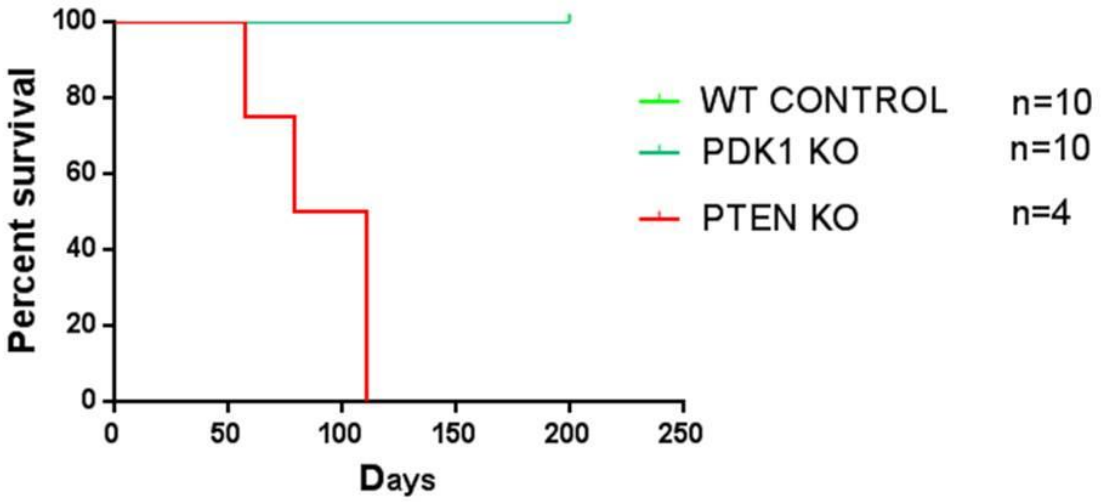


Figure 4.8: PTEN deletion induced ALL.

Graph showing survival of mice after PTEN deletion. (A) Four out of thirteen mice came down with ALL. (B) FACS profile of AML mouse.

Interestingly some of the mice that came down presented with both ALL and AML phenotypes. The mice had slightly enlarged spleen and liver as well as the thymus. Bone marrow examination showed the presence of both T-cells and myeloid cells with the bulk of the myeloid cells positive for both Mac1 and Gr1 in some of the mice. Four out of the thirteen mice that we examined had the presence of both myeloid and T-cells leukaemic cells (Fig 4.9A). The most intriguing finding in some of the mice that came down with AML/ALL was that each organ presented with a different phenotype i.e. AML phenotype in the bone marrow and spleen while the liver presented with ALL phenotype in the same mouse (Fig 4.9B). Three out of the four mice presented with close to 20% of T–cells in bone marrow and more than 50% of T-lineage cells in the liver. One mouse presented with a fewer proportion the T lineage cells in the bone marrow but the liver presented with more than 90% T-lineage cells while the spleen had only 20% T-lineage cells. A classical ALL phenotype was seen in just one mouse that presented with more than 80% of T-lineage cells present in the bone marrow, spleen and liver, in the thymus only T-cells are present as expected.

A


Percent survival
100
80
60
40
20
0
0
50
100
150
200
250
Days
WT CONTROL
PDK1 KO
PTEN KO
n=10
n=10
n=4

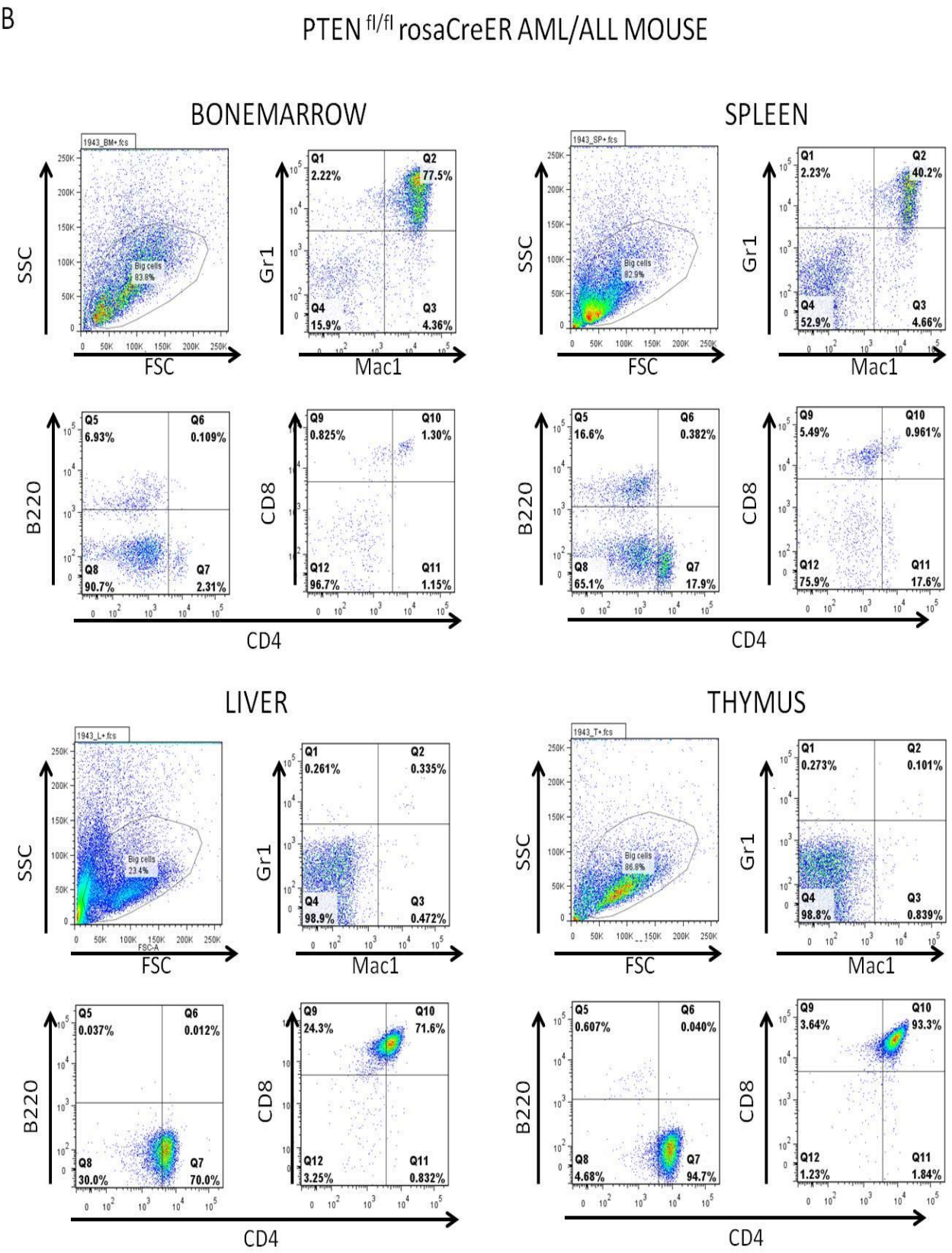


Figure 4.9 PTEN deletion induced AML/ALL.

Graph showing survival of mice after PTEN deletion. (A) Four out of thirteen mice transplanted with PTEN$^{fl/fl}$ Rosa-26cre-ERT presented with AML/ALL. (B) FACS analysis of AML/ALL mouse

4.4 Simultaneous deletion of PTEN and PDK1 resulted only in AML

Surprisingly, mice that were transplant with PTEN$^{fl/fl}$PDK1$^{fl/fl}$Rosa-26cre-ERT did not develop ALL. To specifically examine the delay caused by deletion of PDK1 in PTEN induced AML we show the survival curve for the mice that succumbed to AML (Fig 4.10). Out of the thirteen mice that were transplant with PTEN$^{fl/fl}$Rosa-26cre-ERT cells, only five came down with AML. Six out of ten mice transplanted with PTEN$^{fl/fl}$PDK1$^{fl/fl}$Rosa-26cre-ERT cells succumbed to AML. Remarkably, four mice from the group that received PTEN$^{fl/fl}$PDK1$^{fl/fl}$Rosa-26cre-ERT survived passed 400 day without any symptoms of disease.

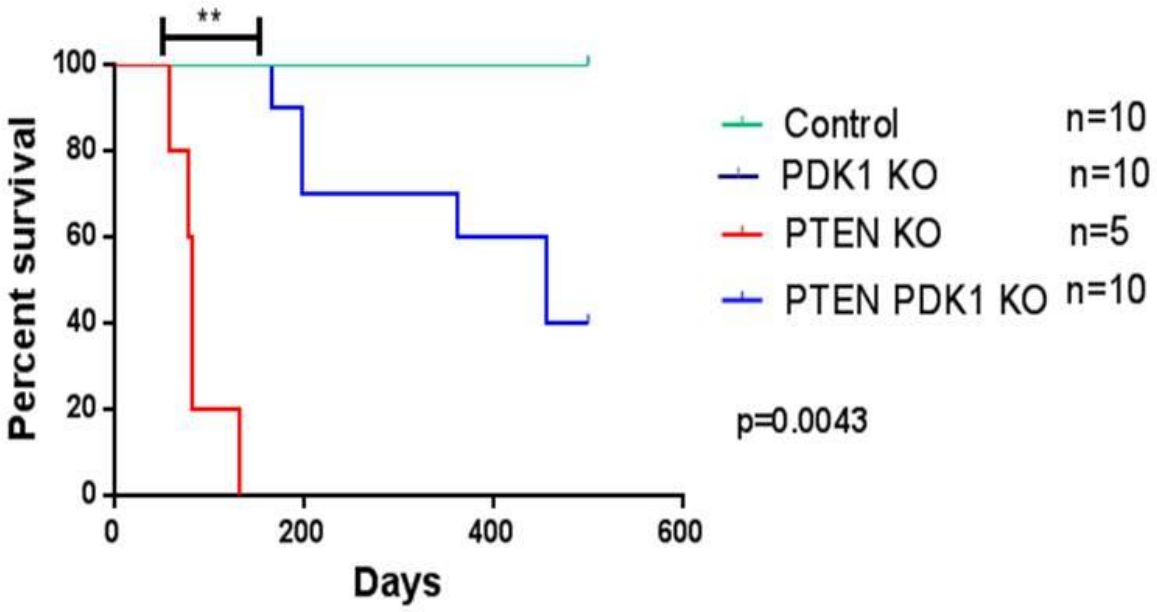
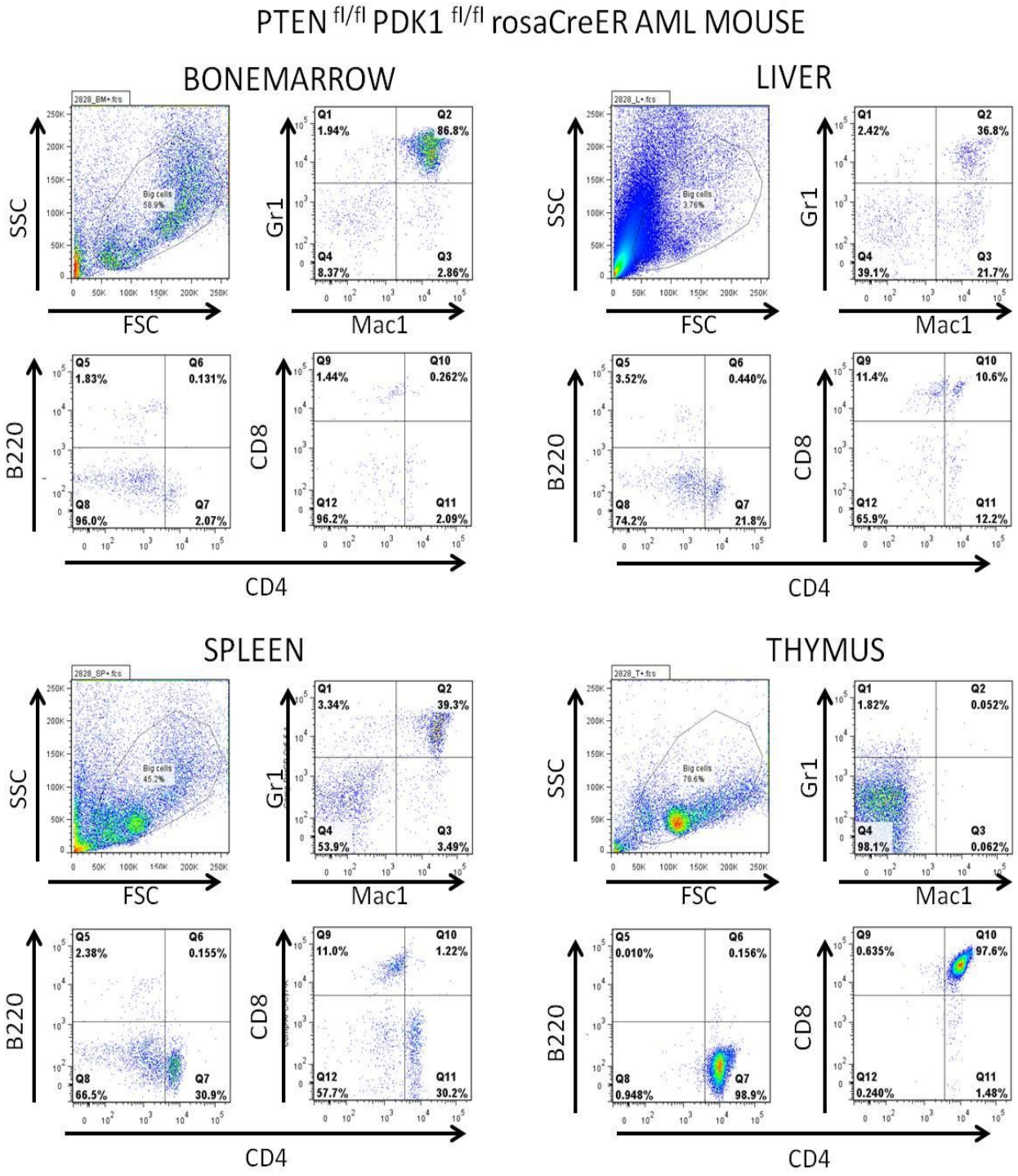


Figure 4.10 Simultaneous deletion PTEN and PDK1 only results in AML.

Graph showing the development of AML in mice transplanted with PTEN$^{fl/fl}$ Rosa-26cre-ERT cells compared to mice transplanted with PDK1$^{fl/fl}$ PTEN$^{fl/fl}$ Rosa-26cre-ERT cells and mice transplanted with wild type cells (P value =0.0043 PTEN$^{fl/fl}$ Rosa-26cre-ERT vs PDK1$^{fl/fl}$ PTEN$^{fl/fl}$ Rosa-26cre-ERT).

FACS analysis of the bone marrow of AML mice showed almost a complete infiltration of the bone marrow with myeloid cells and in most cases more than 80% of the cells were positive for both Mac1 and Gr1 suggesting a shift in the turnover of myeloid cells in the bone marrow in both $PTEN^{fl/fl}$Rosa-26cre-ERT as well as $PTEN^{fl/fl}PDK1^{fl/fl}$ Rosa-26cre-ERT group. The spleen and liver of AML mice also showed increased proportion of myeloid cells with a majority of the cells again positive for both Mac1 and Gr1 (Fig 4.11A). Cell morphology from blood, spleen and bone marrow showed increasing appearance of immature myeloid cells in $PTEN^{fl/fl}$Rosa-26cre-ERT group and $PDK1^{fl/fl}PTEN^{fl/fl}$Rosa-26cre-ERT in all mice that came down with AML (Fig 4.11B). However, the frequency of immature cells in peripheral blood, spleen as well as in the bone marrow was much higher in the mice that received $PTEN^{fl/fl}$Rosa-26cre-ERT cells when compared to mice that received $PDK1^{fl/fl}PTEN^{fl/fl}$Rosa-26cre-ERT cells. Moreover, the size of the immature myeloid cells in AML mice that were transplanted with $PTEN^{fl/fl}$Rosa-26cre-ERT was greater compared to the cells in mice transplanted with $PDK1^{fl/fl}PTEN^{fl/fl}$Rosa-26cre-ERT cells in peripheral blood, spleen and liver. Histology showed infiltration of the liver tissue with blast cells in $PTEN^{fl/fl}$Rosa-26cre-ERT AML mouse as well as $PDK1^{fl/fl}PTEN^{fl/fl}$Rosa-26cre-ERT mouse (Fig 3.11C). Histology of the spleen revealed complete disruption of the splenic architecture when normal spleen is compared to $PDK1^{fl/fl}PTEN^{fl/fl}$Rosa-26cre-ERT AML mouse or $PDK1^{fl/fl}PTEN^{fl/fl}$Rosa-26cre-ERT AML mouse (Fig 4.11D).

A


PTEN fl/fl PDK1 fl/fl rosaCreER AML MOUSE
BONEMARROW
LIVER
SPLEEN
THYMUS
SSC
FSC
Gr1
Mac1
B220
CD8
CD4
Big cells 58.9%
Q1 1.94%
Q2 86.8%
Q4 8.37%
Q3 2.86%
Q5 1.83%
Q6 0.131%
Q8 96.0%
Q7 2.07%
Q9 1.44%
Q10 0.262%
Q12 96.2%
Q11 2.09%
Big cells 3.76%
Q1 2.42%
Q2 36.8%
Q4 39.1%
Q3 21.7%
Q5 3.52%
Q6 0.440%
Q8 74.2%
Q7 21.8%
Q9 11.4%
Q10 10.6%
Q12 65.9%
Q11 12.2%
Big cells 45.2%
Q1 3.34%
Q2 39.3%
Q4 53.9%
Q3 3.49%
Q5 2.38%
Q6 0.155%
Q8 66.5%
Q7 30.9%
Q9 11.0%
Q10 1.22%
Q12 57.7%
Q11 30.2%
Big cells 78.6%
Q1 1.82%
Q2 0.052%
Q4 98.1%
Q3 0.062%
Q5 0.010%
Q6 0.156%
Q8 0.948%
Q7 98.9%
Q9 0.635%
Q10 97.6%
Q12 0.240%
Q11 1.48%

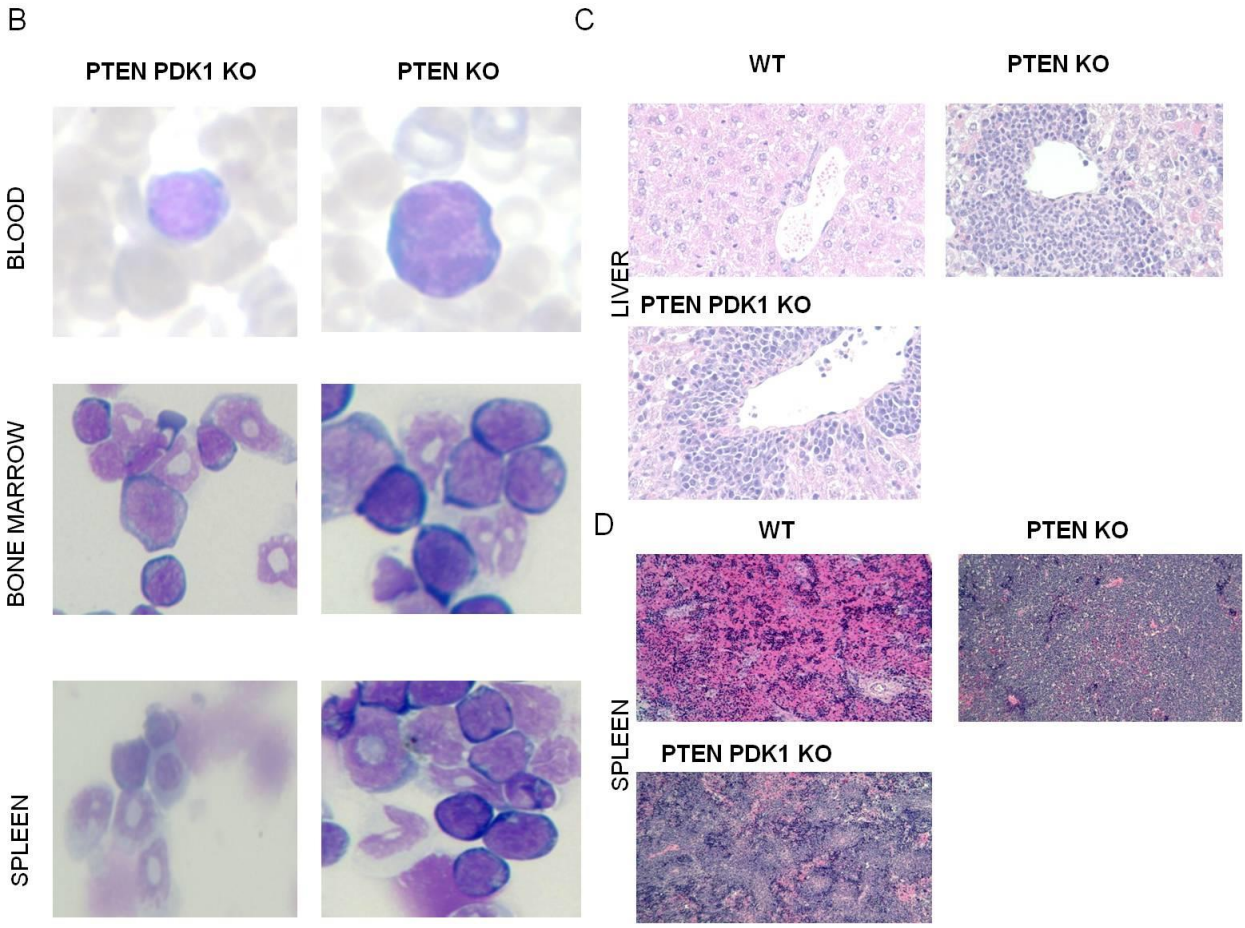


Figure 4.11 PTEN induced AML in mice transplanted with PTEN$^{fl/fl}$ Rosa-26cre-ERT cells and PDK1$^{fl/fl}$ PTEN$^{fl/fl}$ Rosa-26cre-ERT cells

(A) FACS profile from a mouse transplant with PDK1$^{fl/fl}$ PTEN$^{fl/fl}$ Rosa-26cre-ERT cells that succumbed to AML. (B) Cell morphology from AML mice blood film from PDK1$^{fl/fl}$ PTEN$^{fl/fl}$ Rosa-26cre-ERT mouse group vs blood film from PTEN$^{fl/fl}$ Rosa-26cre-ERT mouse group. Bone marrow cytospin from PDK1$^{fl/fl}$ PTEN$^{fl/fl}$ Rosa-26cre-ERT mouse group vs bone marrow cytospin from PTEN$^{fl/fl}$ Rosa-26cre-ERT mouse group. Spleen cytospin from PDK1$^{fl/fl}$ PTEN$^{fl/fl}$ Rosa-26cre-ERT mouse group vs spleen cytospin from PDK1$^{fl/fl}$ PTEN$^{fl/fl}$ Rosa-26cre-ERT mouse group. (C) Infiltration of the liver with blast cells (compare WT to PTEN KO and PTEN PDK1 KO). (D) Complete effacement of the splenic architecture (compare WT to PTEN KO and PTEN PDK1 KO).

4.4.1 PTEN loss induced AML is independent from PDK1 signalling.

During normal PI3K pathway signalling, PDK1 is required for activation of several effector molecules such as AKT, RSK and PKC fundamental to cell survival, growth and migration (Bayascas, 2010, Gagliardi et al., 2015, Hossen et al., 2015). Lack of

PDK1 in mouse embryonic stem (ES) cells resulted in failure to activate PKB, S6K and RSK when exposed to stimuli that trigged the activation of these enzymes in wild-type ES cell (Balendran et al., 2000). Thus, we aimed to investigate the mechanism of leukaemogenesis mediated by loss of PTEN in the absence of PDK1. Total bone marrow cells from mice transplanted with $PTEN^{fl/fl}$Rosa-26cre-ERT and $PDK1^{fl/fl}PTEN^{fl/fl}$Rosa-26cre-ERT cells that developed AML were analysed to evaluate the role of PTEN and PDK1 in the PI3Kinase signalling pathway during leukaemogenesis. We used PDK1 KO cells generated by treating mononuclear cells $PDK1^{fl/fl}$ Rosa-26cre-ERT with 300 nM of tamoxifen and wild type cells from a healthy C57BL6 as controls to demonstrate the normal function of PTEN and PDK1. Bone marrow cells were not exposed to any stimuli to activate PI3K pathway enzymes prior to preparing cell lysates. We demonstrate that leukemic cells isolated from the bone marrow of mice transplanted with $PTEN^{fl/fl}$Rosa-26cre-ERT have constitutively high levels of phosphorylated AKT at Thr 308 and Ser473 as well as SGK1 Thr256 (Fig 3.12). Interestingly, high levels of phosphorylated AKT Thr308 and SGK1 Thr256 were observed in leukemic cells isolated from mice transplanted with $PDK1^{fl/fl}PTEN^{fl/fl}$Rosa-26cre-ERT. While under normal conditions PDK1 is the only known kinase responsible for the activation of AKT at Thr308, our findings suggest that PTEN deletion mediated AML cells may possess an alternative pathway that bypasses the normal requirement for PDK1 to mediate the phosphorylation of downstream kinases. These results suggest that PTEN null leukemic cells can grow, proliferate, survive and possess pAKT (Thr308) suggesting an uncompromised PI3K

pathway even in the absence of PDK1. This result also suggest that AKT ativation may still be a requirement for the survival of AML cells.

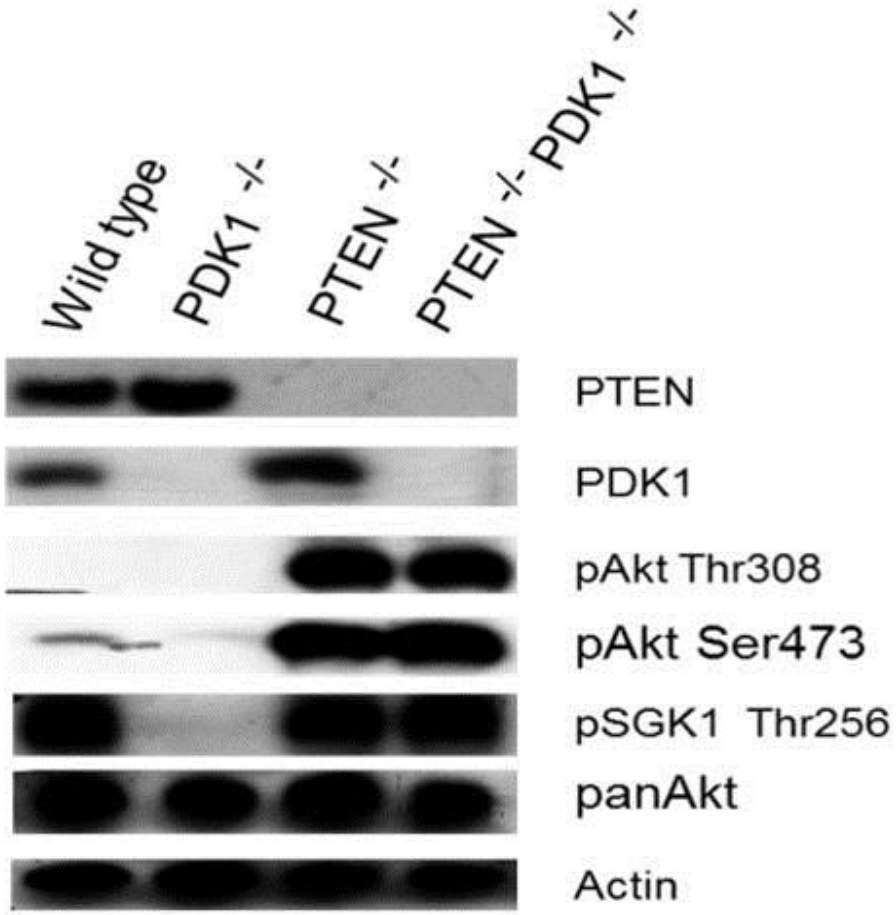


Figure 4.12 PTEN deleted AML cells by passes the normal requirement of PDK1 to activate downstream enzymes.

Western blot analysis of AKT Thr308, Ser473 and SGK1 Thr256 phosphorylation in $PTEN^{fl/fl}$ Rosa-26cre-ERT and $PDK1^{fl/fl}$ $PTEN^{fl/fl}$ Rosa-26cre-ERT bone marrow leukaemic cells.

4.4.2 PTEN deficient AML cells failed to induce leukaemia in secondary recipient mice

The functionality of leukaemia stem cells is determined by their ability to induce leukaemia in secondary recipient mice. Leukaemia-initiating cells are able to maintain

diseases upon serial transplant (Lapidot et al., 1994). To test whether PTEN deficient AML cells and PTEN PDK1 deficient AML cells can be transplantable and transfer AML to irradiated mice we transplanted bulk AML cells isolated from mice that came down with AML after transplant with $PTEN^{fl/fl}$Rosa-26cre-ERT cells and $PDK1^{fl/fl}PTEN^{fl/fl}$ Rosa-26cre-ERT cells. Transplanting the bulk leukaemia cells did not transfer the disease to secondary recipient mice with both genotypes in my hands and all mice survived past 50 day without any symptoms of leukaemia (Fig 4.13). This findings are contradictory to previous finding by Yilmaz that suggested PTEN deficient AML can be transplantable in secondary recipient mice (Yilmaz et al., 2006b).

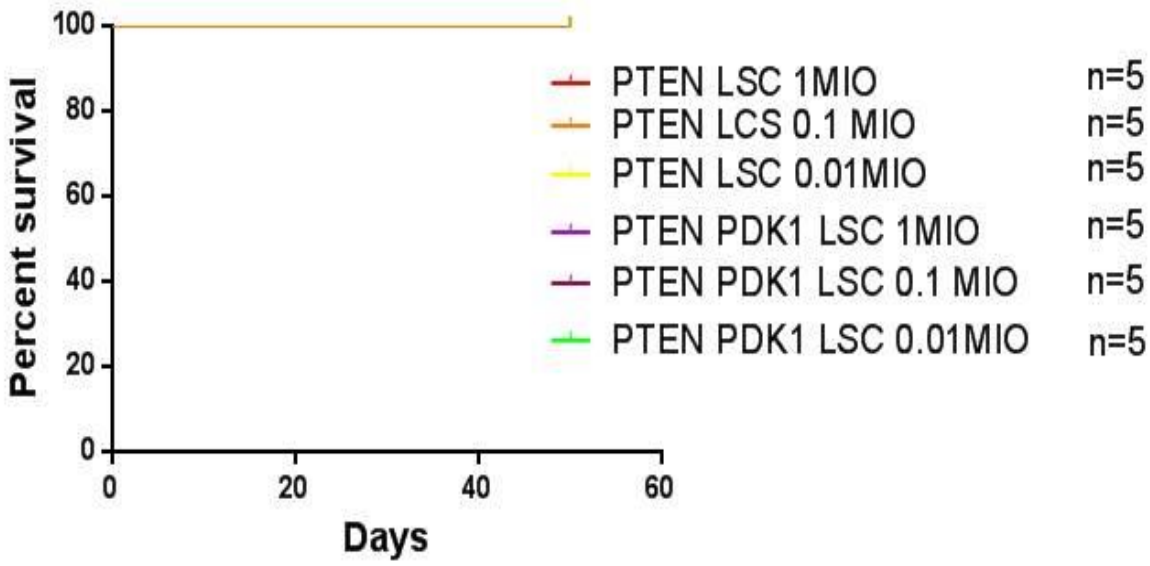
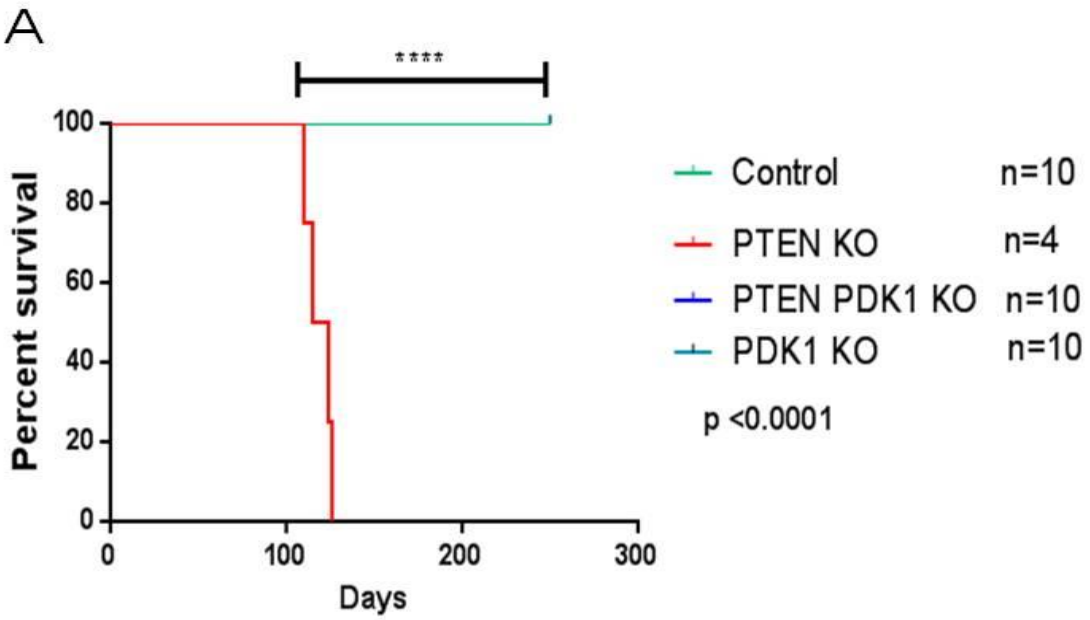


Figure 4.13 PTEN KO AML cells and PTEN PDK1 KO AML cells failed to transferee disease to secondary mice

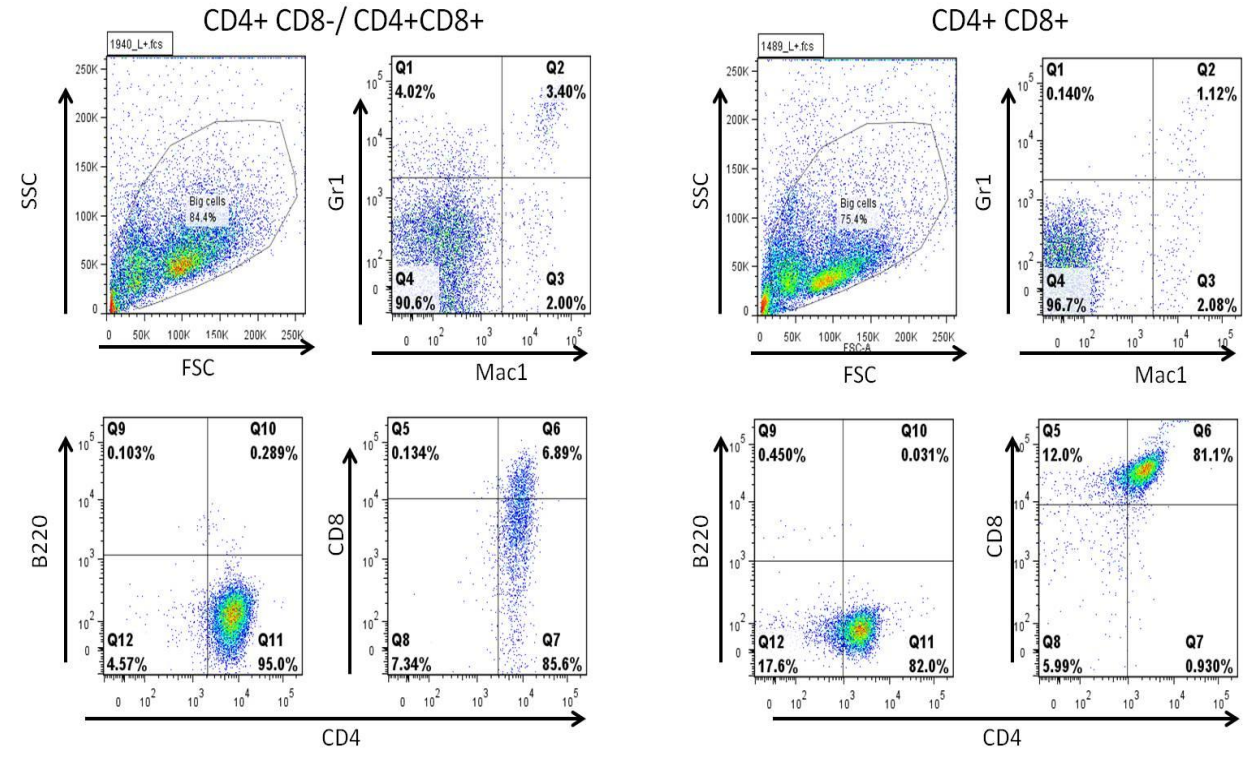
Graph showing survival of mice after transplant win PTEN deficient AML cells vs PTEN PDK1 deficient AML cells (1 mio n=5, 0.1 mio n=5, 0.01 mio n=5).

4.4.3 PTEN deletion induced T-ALL tumour cells appeared to be at different stages in each mouse

As described above, 30 % of mice develop ALL upon PTEN deletion. ALL cells showed high expression of CD4 and/or CD8 in the bone marrow, spleen, liver and thymus and were accordingly classified as T-ALL. Strikingly, this phenotype was found only in mice that received $PTEN^{fl/fl}$Rosa-26cre-ERT cells whereas none of the mice that received $PDK1^{fl/fl}$ $PTEN^{fl/fl}$ Rosa-26cre-ERT showed the presence of these clonal cells in the bone marrow, spleen or liver of mice that succumbed to illness (Fig 4.14A). A closer examination of the liver for the origin of the T-lineage cells using CD8 and CD4 markers revealed that the tumour cells were at different stages in each mouse. Three out of four (50%) mice with T cell tumour showed to have majority of the cells positive only for CD4 ($CD4^+CD8^-$) with a small portion of the cells double positive CD4 and CD8 cells ($CD4^+CD8^+$) (Fig 4.14B). The other two mouse were found to have majority of the T cells double positive CD4 and CD8 ($CD4^+CD8^+$). We further characterised the tumour cells using May Grunwald and Giemsa stain. Peripheral blood smear prepared using blood drawn from heart puncture revealed the presence of ALL blast in circulation in mice that succumb to T-ALL (Fig4.14C). These cells appeared to be larger compared to normal mature lymphocytes. Cytospin from bone marrow and spleen from the ALL mice also revealed the presence of ALL blast accounting for more than 50% of the total bone marrow and spleen cells. Together these data suggest that PTEN deletion might induce T-ALL which can originate from double positive $CD4^+$ and $CD8^+$ thymocytes.


A
****
Percent survival
100
80
60
40
20
0
0
100
200
300
Days
Control n=10
PTEN KO n=4
PTEN PDK1 KO n=10
PDK1 KO n=10
p <0.0001

B


CD4+ CD8-/ CD4+CD8+
CD4+ CD8+
SSC
FSC
Big cells 84.4%
Big cells 75.4%
Gr1
Mac1
Q1 4.02%
Q2 3.40%
Q4 90.6%
Q3 2.00%
Q1 0.140%
Q2 1.12%
Q4 96.7%
Q3 2.08%
B220
CD8
CD4
Q9 0.103%
Q10 0.289%
Q12 4.57%
Q11 95.0%
Q5 0.134%
Q6 6.89%
Q8 7.34%
Q7 85.6%
Q9 0.450%
Q10 0.031%
Q12 17.6%
Q11 82.0%
Q5 12.0%
Q6 81.1%
Q8 5.99%
Q7 0.930%

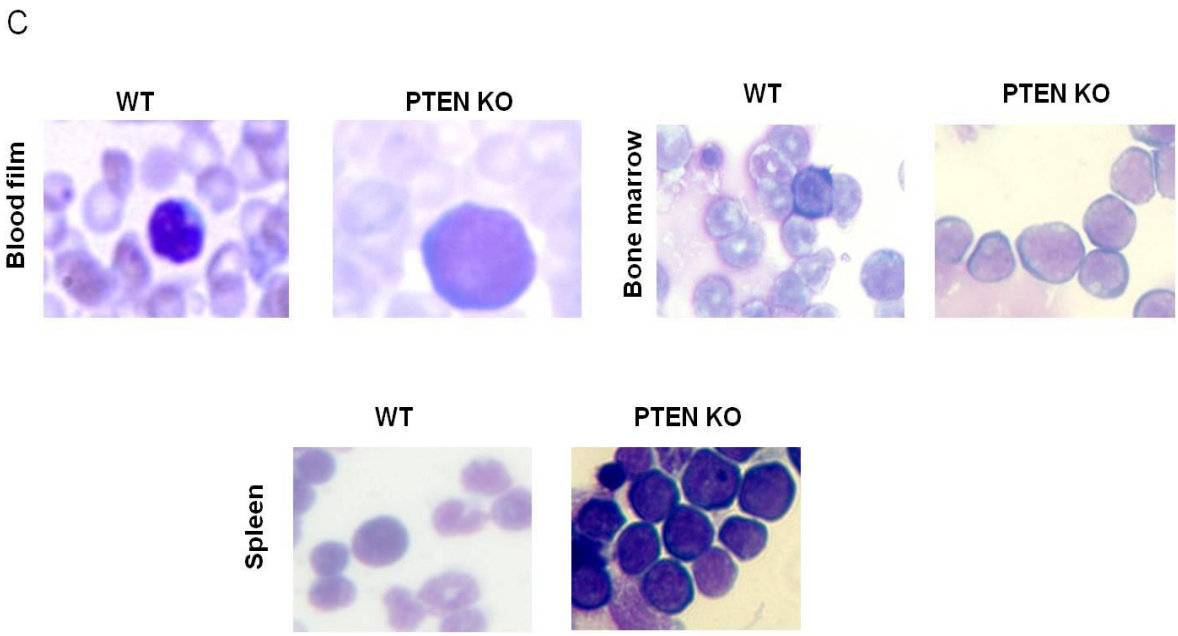


Figure 4.14 PTEN deletion induce ALL.

Graph showing the development of ALL in mice transplanted with PTEN$^{fl/fl}$ Rosa-26cre-ERT cells compared to mice transplanted with PDK1$^{fl/fl}$ PTEN$^{fl/fl}$ Rosa-26cre-ERT cells and mice transplanted with wild type cells (P value > 0.0001 PTEN$^{fl/fl}$Rosa-26cre-ERT vs PDK1$^{fl/fl}$ PTEN$^{fl/fl}$Rosa-26cre-ERT). (B) FACS profile of ALL liver from mice transplanted with PTEN$^{fl/fl}$Rosa-26cre-ERT.

4.4.4 PTEN deficient T-ALL cells are able to engraft and induce secondary leukaemia

To test the ability of PTEN null ALL cells to engraft and induce secondary leukaemia, we set up a secondary transplant experiment using cells isolated from mice that were transplanted with PTEN$^{fl/fl}$ Rosa-26cre-ERT cells that succumbed to T-ALL. To determine the frequency of PTEN null T-ALL stem cells required to induce a secondary T-ALL in recipient mice we transplanted 1 mio, 0.1 mio, and 0.01 mio into groups of 5 mice per cohort. We demonstrate that PTEN null T-ALL cells were able

to induce leukaemia in secondary recipient mice in less than 30 days when 1 mio or 0.1 mio were transplanted whereas mice that receive 0.01Mio cells where able to survive past 60 days without and symptoms of illness. The phenotype of the secondary leukaemia showed the presence of double positive CD4 and CD8 lymphocytes similar to the primary leukaemia (Fig 4.15). Our findings are consistent with previous data from that suggested that PTEN deficient ALL cells are transplantable (Yilmaz et al., 2006b).

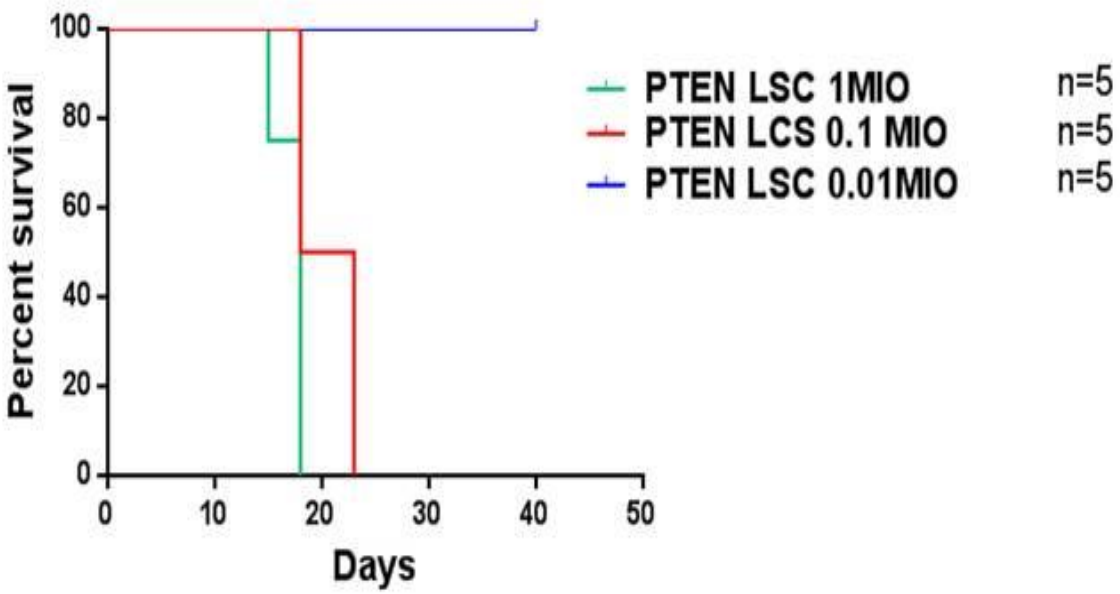


Figure 4.15Limiting dilution transplant of PTEN KO ALL cells

Graph showing the development of secondary ALL in mice transplanted with different numbers of PTEN null ALL cells (1 mio n=5, 0.1 mio n=5, 0.01 mio n=5).

4.5 PDK1 MGK465 mutation has little influence on the PTEN deletion phenotype

Given that loss of PDK1 rescues or changes the phenotypes observed upon loss of PTEN, we sought to investigate which property of PDK1 mediates this response. To further elucidate the role of PDK1 in loss of PTEN mediated leukaemogenesis, we induced PTEN deletion in $PDK1^{MGK465/MGK465}$ cells.

PDK1$^{MGK465/MGK465}$ mutant bears a mutation that disrupt the normal fuction of the PH domain of the PDK1 Protein therefore distorts its ability to phosphorylate AKT Thr308. To this end, transplantation experiments with PDK1$^{MGK465/MGK465}$PTEN$^{fl/fl}$ Rosa-26cre-ERT cells were carried out. The engraftment levels four weeks after transplantation were around 60% which is comparable with other experiments. Interestingly, similar to PTEN deletion, deletion of PTEN in the PDK1 MGK465 background led to an increase in engraftment levels over time (Fig 4.16C). Moreover, an increase in Mac1/Gr1 positive cells was observed at week 4, 8, and most dramatically at week 12, whereas the Mac1 positive cells were increased at weeks 4, and 8 but not at week 12. On the other hand, a reduction of B220 B-cells was observed. The observed phenotype of the PDK1$^{MGK465/MGK465}$ PTEN deletion closely resembles that of PTEN deletion suggesting that PDK1 MGK465 has little influence on the PTEN deletion phenotype (Fig 4.16D). While the control group with PDK1$^{MGK465/MGK465}$ without PTEN deletion also showed an increase of engraftment levels over time, (Fig 4.16A), the distribution of myeloid and lymphoid cells also changed over time as an decrease of both Mac1/Gr1 and Mac1 positive myeloid cells and an increase of B220 B-cells as well as CD4 and CD8 T- cells was observed (Fig 4.16B) . To confirm the deletion of PTEN and presence of floxed mutant allele we isolated DNA from blood samples four weeks post cre-activation. We show that PTEN was completeley deleted indicated by the absent of the floxed band (Fig 4.16E) . We also confirm the presense of the MGK465E allele by showing the presense of a floxed band (Fig 4.16F).

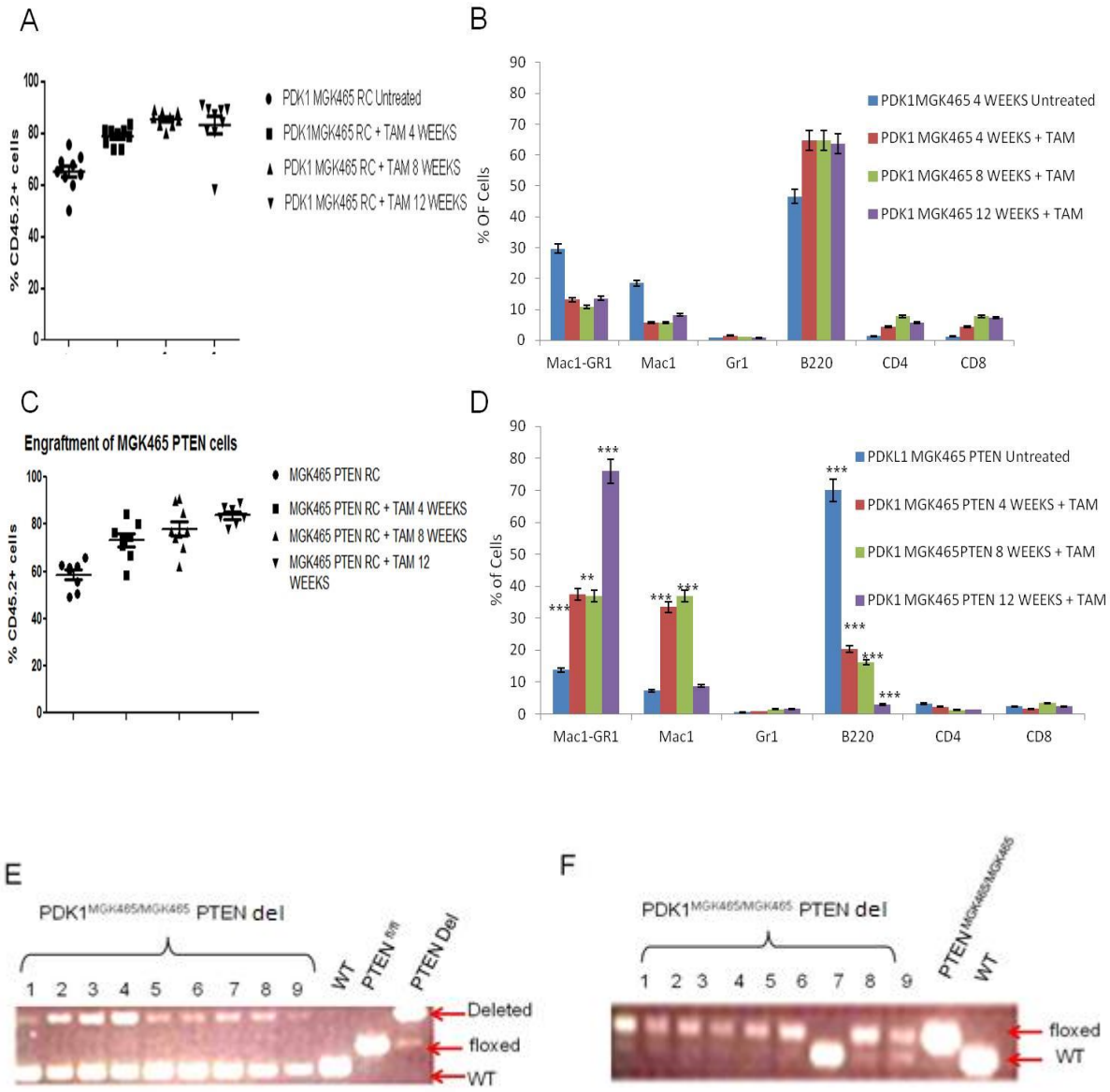


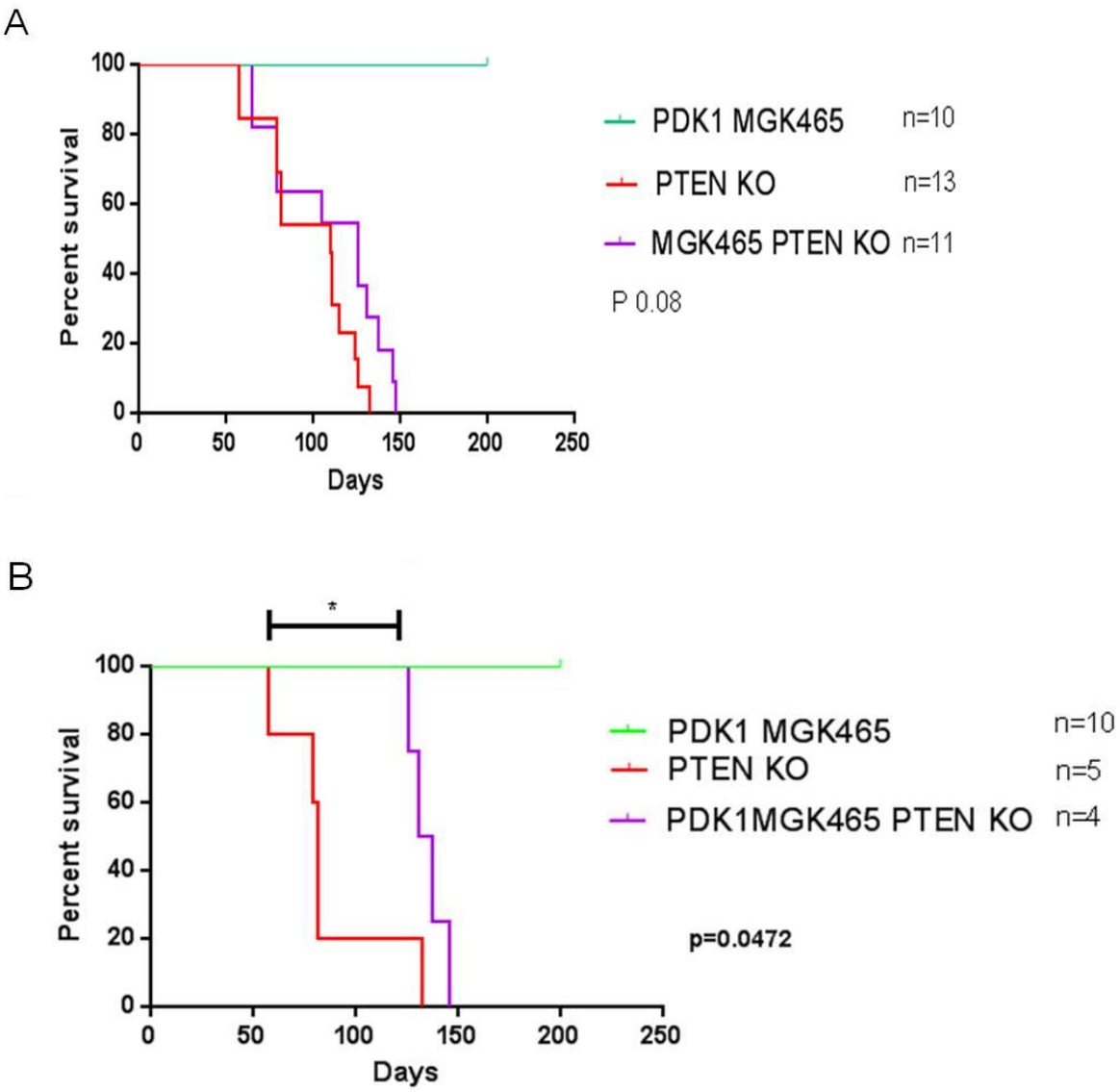
Figure 4.16 Reconstitution with PDK1 $^{MGK465/MGK465}$PTEN $^{fl/fl}$ Rosa-26cre-ERT cells.

PDK1$^{MGK465/MGK465}$PTEN$^{fl/fl}$ Rosa-26cre-ERT cells and PDK1$^{MGK465/MGK465}$ (CD45.2 1 Mio cells) were transplanted into SJL mice (n=10) (CD 45.1 0.2 mio rescue cells). (A) and (C) The percentage of CD45.2 donor cells 4 weeks after transplantation and every 4 weeks after tamoxifen treatment is show. (B) and (D) Immunophenotying of donor cells before and after tamoxifen with treatment is shown. Significance (* P <0.05 ** P >0.005 *** P > 0.001). (E) PCR showing complete deletion of PTEN. (F) PCR showing the presence of floxed PDK1$^{MGK465/MGK465}$ mutant allele.

4.5.1 PDK1 MGK465 mutation has little influence on the PTEN mediated leukeamogenesis

We investigated the specific function of PDK1 by observing the latency of leukaemia induced by PTEN deletion in PDK1 MGK465 mutant cells. Surprisingly, the PDK1 MGK465 mutation had little effect on the general onset of leukaemia in mice transplanted with $PDK1^{MGK465/MGK465}PTEN^{fl/fl}$Rosa-26cre-ERT cells compared to mice that received $PTEN^{fl/fl}$ Rosa-26cre-ERT cells. All mice from each group died between 100 to 150 days post tamoxifen treatment (fig 4.17A). Consequently, both AML and ALL phenotype were observed in mice transplanted with $PDK1^{MGK465/MGK465}PTEN^{fl/fl}$ Rosa-26cre-ERT cells similar to what was observed in mice transplant with $PTEN^{fl/fl}$ Rosa-26cre-ERT cells. However, when we assesed the latency of AML alone and compared between mice transplanted with $PDK1^{MGK465/MGK465}PTEN^{fl/fl}$Rosa-26cre-ERT cells vs mice transplant with $PTEN^{fl/fl}$ Rosa-26cre-ERT cells, we noted a slight significant delay on the onset of illness between the two groups (Fig 4.17B). Cell morphology from blood collected from the heartrevealed the presense of myeloid blast in peripheral blood. An increase in the frequency of myeloid blasts was also observed in the bone marrow and spleen (Fig 4.17C). FACS data showed no difference in the phenotype of AML cells between the two groups. AML was characterized by expression of double positive Mac1/Gr1 cells making over 80% of the bone marrow population. The double positive Mac1 and Gr1 phenotype was also found to be the major fractions in the spleen and liver (Fig 4.17D). Given that the PDK1 MGK465 mutation disrupts the PH domain responsible for recruiting and phosphorylating AKT at the membrane, we therefore speculate slight delay on the onset leukamia might be a result deficient or delayed AKT activation

suggestng AKT might have a signicant role in AML cause by loss of function o the PTEN protein. These results support data from other studies that showed inhibition of AKT inhibited growth and induced apoptosis in AML cell lines and primary AML blasts (Sujobert et al., 2005).



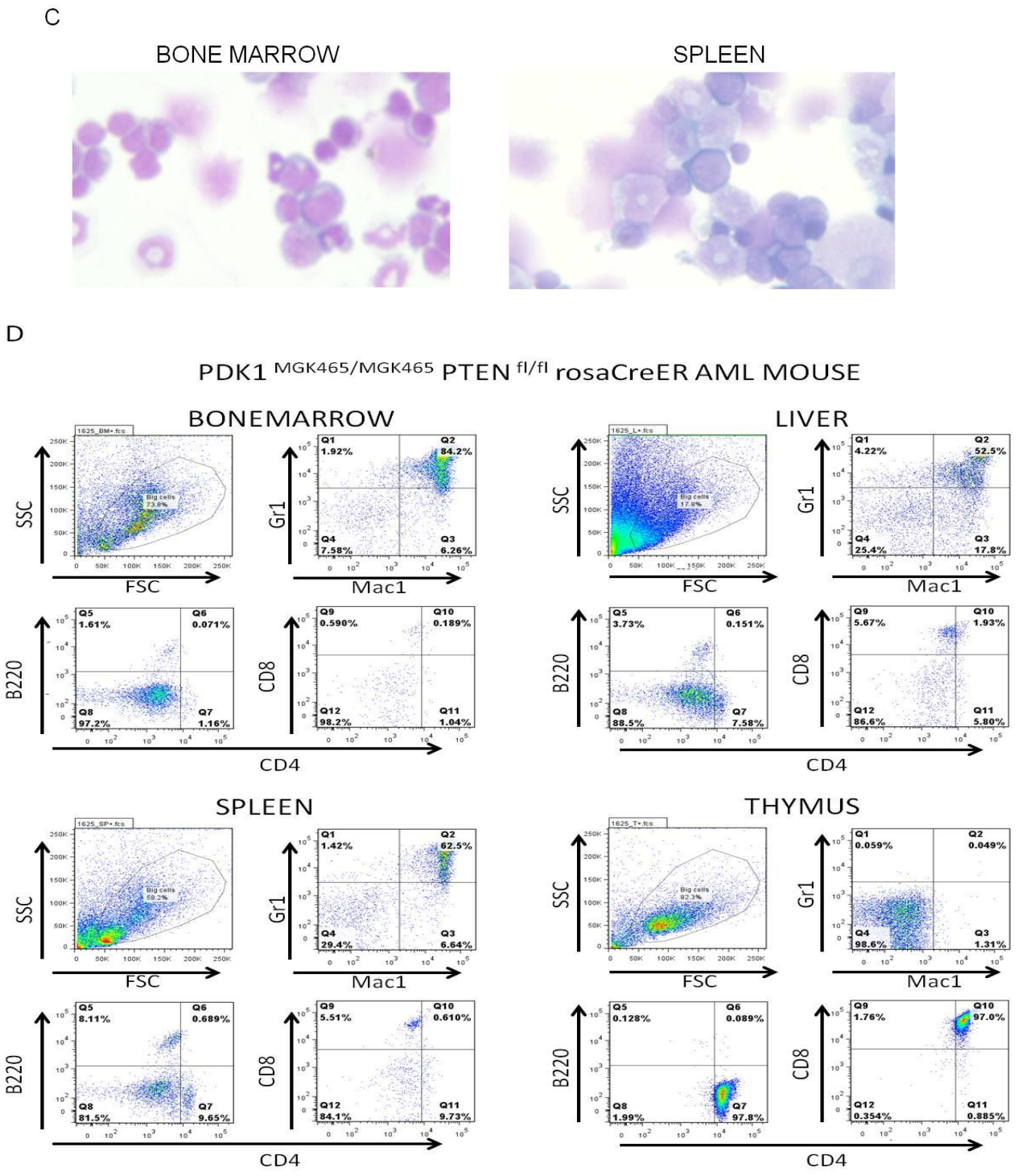


Figure 4.17 PDK1$^{MGK465/MGK465}$ mutation slightly delayed the onset of PTEN deletion mediated AML.

(A) Graph showing the development of leukaemia in mice transplanted with PTEN$^{fl/fl}$Rosa-26cre-ERT cells compared to mice transplanted with PDK1$^{MGK465/MGK465}$PTEN$^{fl/fl}$Rosa-26cre-ERT cells and mice transplanted with PDK1$^{MGK465/MGK465}$ cells. (B) Graph showing the development of AML in mice transplanted with PTEN$^{fl/fl}$Rosa-26cre-ERT cells compared to mice transplanted with PDK1$^{fl/fl}$PTEN$^{fl/fl}$Rosa-26cre-ERT cells and mice transplanted with PDK1$^{MGK465/MGK465}$ cells (P value =0.0472 PTEN$^{fl/fl}$Rosa-26cre-ERT vs PDK1$^{MGK465/MGK465}$PTEN$^{fl/fl}$Rosa-26cre-ERT). (C) Cell morphology from bone marow and spleen of AML mouce transplanted with PTEN$^{fl/fl}$Rosa-26cre-ERT

cells. (D) FACS profile of AML mice transplanted with PDK1$^{MGK465/MGK465}$ PTEN$^{fl/fl}$Rosa-26cre-ERT cells.

4.5.2 PDK1 MGK 465 mutation has no effect on AKT signalling in PTEN null AML cells

To gain further insight into the function of PDK1 using the PDK1 MGK465 mutation, we isolated bone marrow cells from mice transplanted with PDK1$^{MGK465/MGK465}$PTEN$^{fl/fl}$Rosa-26cre-ERT cells and PTEN$^{fl/fl}$Rosa-26cre-ERT cells. Our earlier data showed that PTEN null AML cells isolated from bone marrow of mice transplant with PTEN$^{fl/fl}$Rosa-26cre-ERT cells had elevated levels of phosphorylated AKT both at Thr308 and Ser473 as well as on SGK1 Ser256. Interestingly we observed that elevated levels of phosphorylated AKT Thr308 and Ser 473 were observed in cells isolated from mice transplant with PDK1$^{MGK465/MGK465}$PTEN$^{fl/fl}$Rosa-26cre-ERT cells (Fig 4.17). We previously showed that deletion of PDK1 in PTEN null leukaemic cells failed to inhibit the phosphorylation of down stream PDK1 targets. These results further supports the idea that deletion of PTEN bypass the normal requirement of PDK1 to activate down stream targets such as AKT in AML.

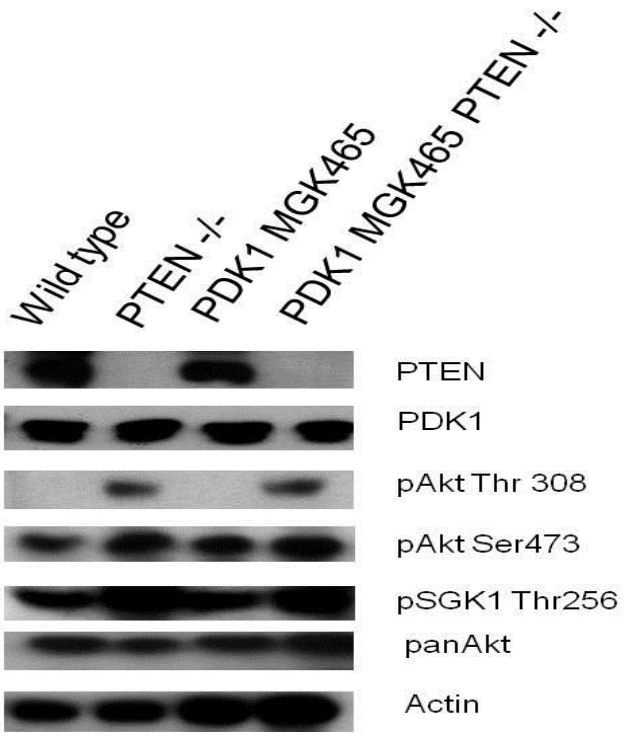


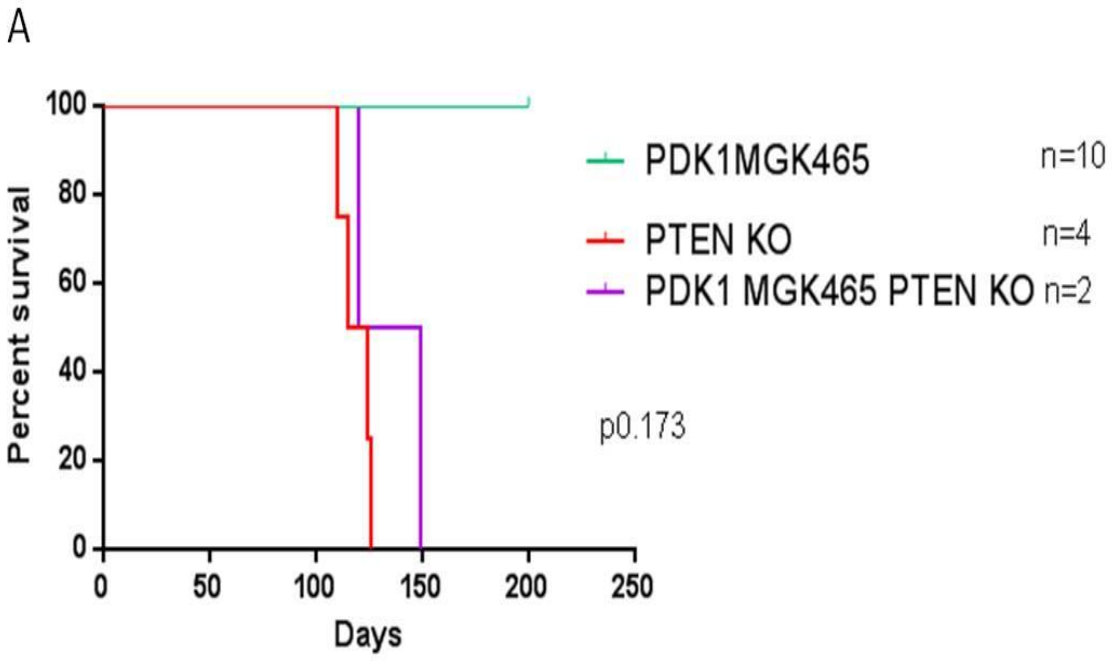
Figure 4.18 PDK1 MGK465 mutation has no effect on AKT Thr 308 in PTEN null AML cells.

Western blot analysis of AKT Thr308, Ser473 and SGK1 Thr256 phosphorylation in $PTEN^{fl/fl}$Rosa-26cre-ERT and $PDK1^{fl/fl}PTEN^{fl/fl}$Rosa-26cre-ERT bone marrow leukaemic cells.

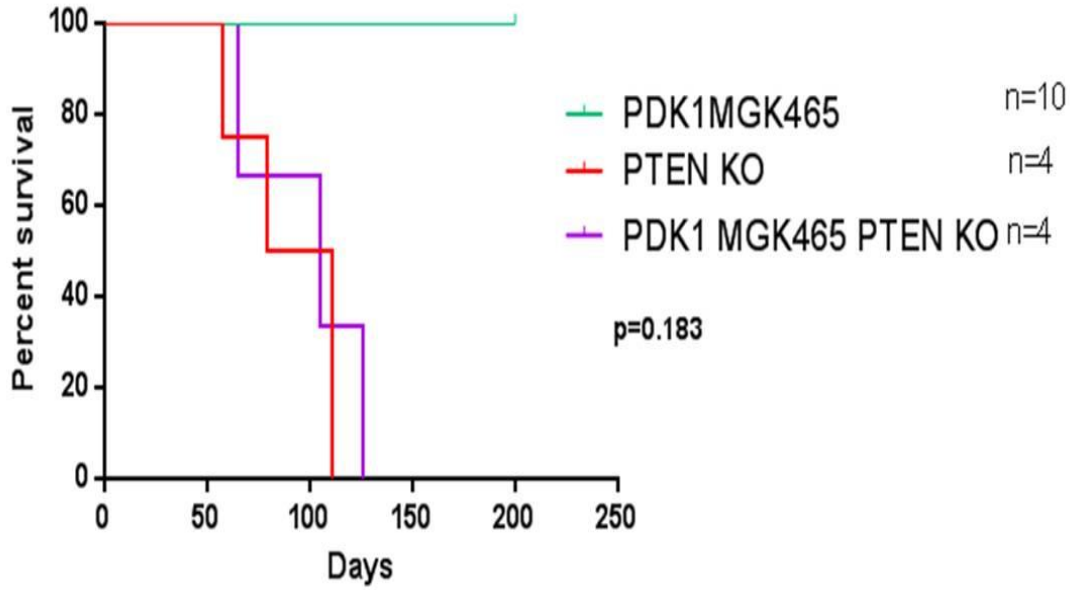
4.5.3 PTEN deletion induced ALL and AML/ALL phenotypes in PDK1 MGK465 mutant

PDK1 MGK465 mutation did not seem to have much effect on PTEN deletion induced ALL as compared to the simultaneous deletion of PTEN and PDK1, which resulted in significant delay of leukaemia and complete elimination of ALL. We compared the latency of ALL and AML/ALL in mice transplant $PTEN^{fl/fl}$Rosa-26cre-ERT cells to mice transplant with $PDK1^{MGK465/MGK465}PTEN^{fl/fl}$Rosa-26cre-ERT cells. Mice in both groups developed ALL or AML/ALL and died between 100 to 150 days after PTEN deletion with no significant difference on the onset of illness. Only two mice out of

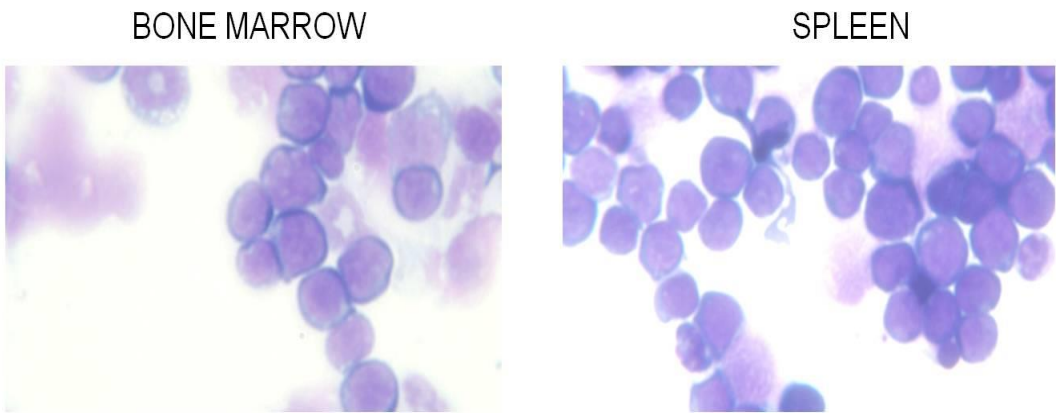
ten mice transplanted with PDK1$^{MGK465/MGK465}$PTEN$^{fl/fl}$Rosa-26cre-ERT cells succumbed to ALL while four of the mice succumbed to AML/ALL (4.19 A and B). FACS analysis of the liver revealed that the T-lineage leukemic cells found in one of the ALL mice were mostly double positive for CD4 and CD8 ($CD4^+CD8^+$) (Fig 4.19D) while the other mouse showed to have majority of the cells positive for CD4 and negative for CD8 ($CD4^+CD8^-$). Cell morphology from peripheral blood revealed the presence of lymphoid blasts in circulation. Increased frequency of lymphoblast was also observed in spleen and bone marrow (Fig 4.19C). FACS profile of mice that came down with AML/ALL showed expansion of double positive Mac1 and Gr1 myeloid cells ($Mac1^+Gr1^+$) in the bone marrow while the liver or spleen showed to have expansion of double positive CD4 and CD8 cells ($CD4^+CD8^+$) (Fig 4.19E).



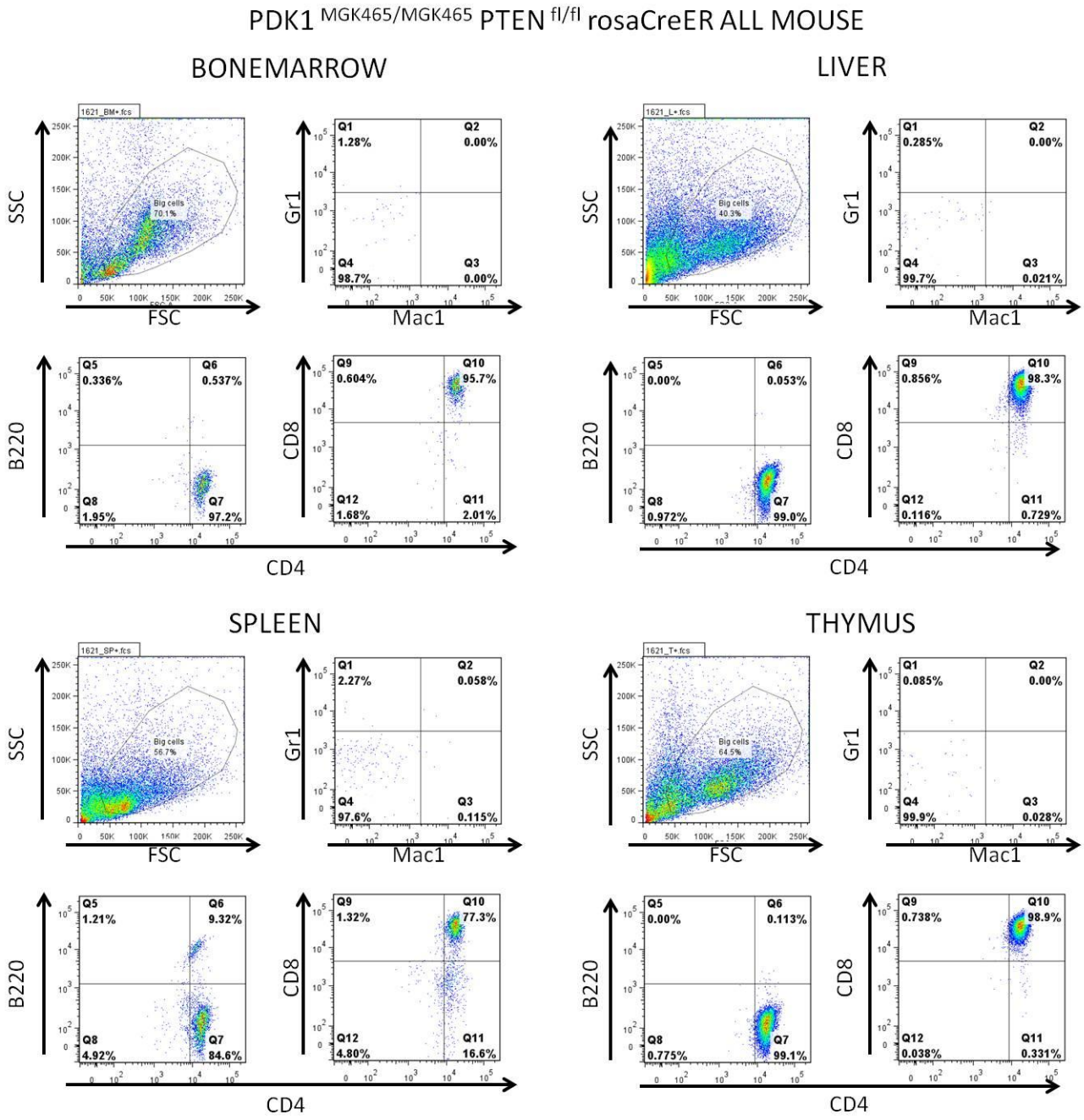
B


Percent survival
100
80
60
40
20
0
0
50
100
150
200
250
Days
PDK1MGK465
PTEN KO
PDK1 MGK465 PTEN KO
n=10
n=4
n=4
p=0.183

C


BONE MARROW
SPLEEN

D


PDK1 MGK465/MGK465 PTEN fl/fl rosaCreER ALL MOUSE
BONEMARROW
LIVER
SPLEEN
THYMUS
SSC
FSC
Gr1
Mac1
B220
CD4
CD8
Big cells 70.1%
Q1 1.28%
Q2 0.00%
Q4 98.7%
Q3 0.00%
Q5 0.336%
Q6 0.537%
Q8 1.95%
Q7 97.2%
Q9 0.604%
Q10 95.7%
Q12 1.68%
Q11 2.01%
Big cells 40.3%
Q1 0.285%
Q2 0.00%
Q4 99.7%
Q3 0.021%
Q5 0.00%
Q6 0.053%
Q8 0.972%
Q7 99.0%
Q9 0.856%
Q10 98.3%
Q12 0.116%
Q11 0.729%
Big cells 56.7%
Q1 2.27%
Q2 0.058%
Q4 97.6%
Q3 0.115%
Q5 1.21%
Q6 9.32%
Q8 4.92%
Q7 84.6%
Q9 1.32%
Q10 77.3%
Q12 4.80%
Q11 16.6%
Big cells 64.5%
Q1 0.085%
Q2 0.00%
Q4 99.9%
Q3 0.028%
Q5 0.00%
Q6 0.113%
Q8 0.775%
Q7 99.1%
Q9 0.738%
Q10 98.9%
Q12 0.038%
Q11 0.331%

E

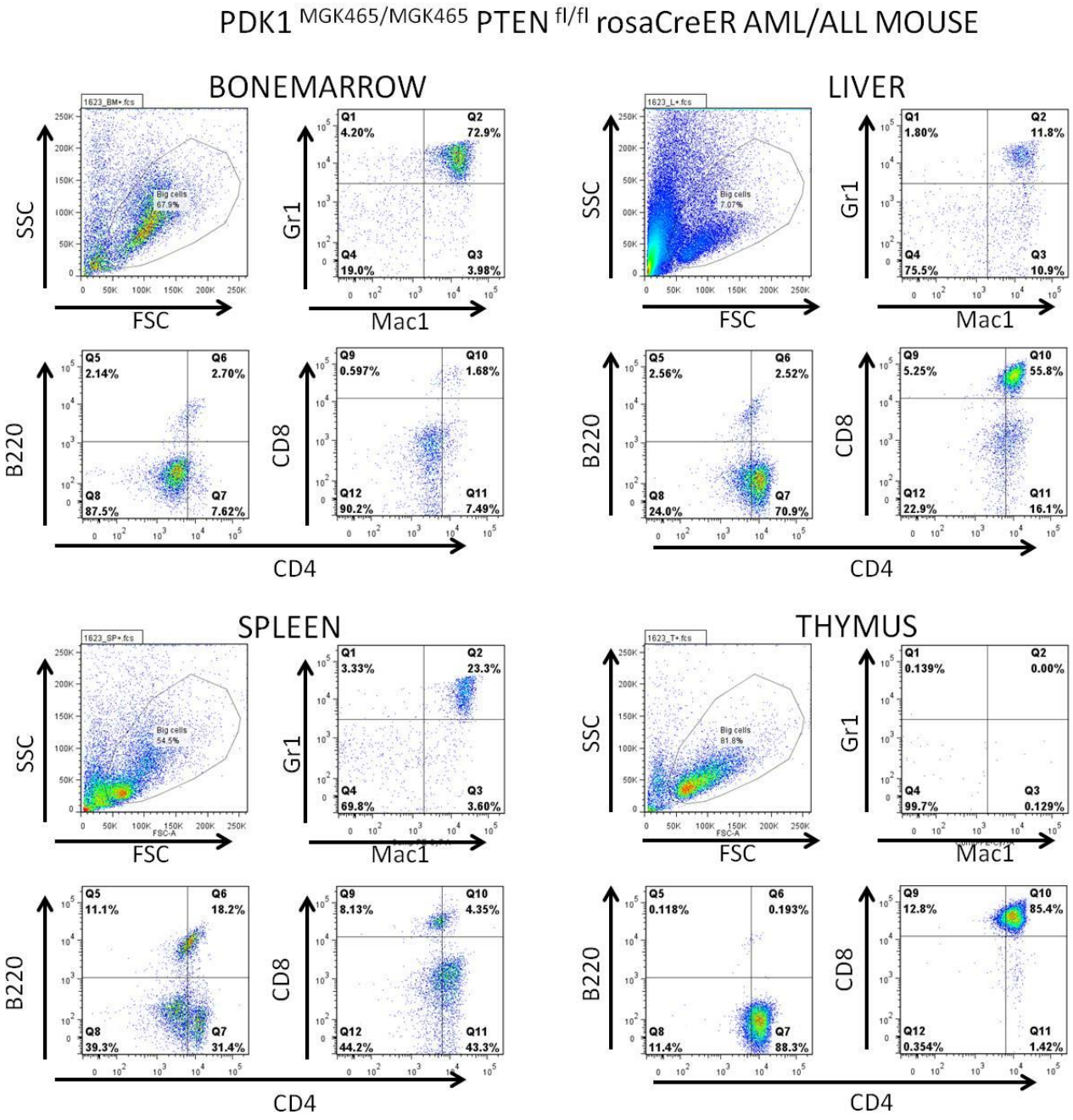
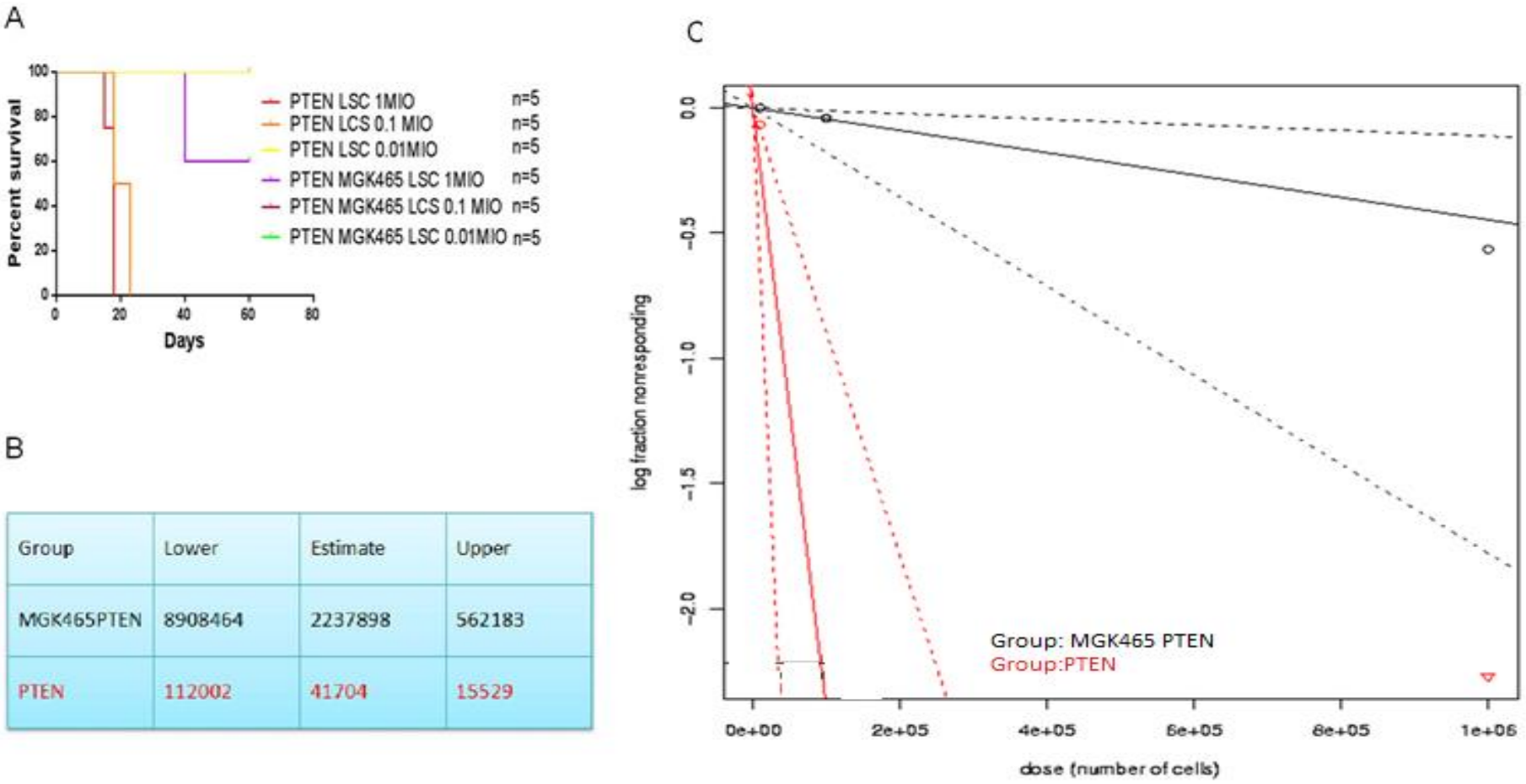


Figure 4.19 PTEN deletion induced ALL and AML/ALL phenotypes in PDK1 MGK465 mutant.

(A) Graph showing the development of ALL in mice transplanted with PTENfl/flRosa-26cre-ERT cells compared to mice transplanted with PDK1$^{MGK465/MGK465}$PTENfl/flRosa-26cre-ERT cells. (B) Graph showing the development of AML/ALL in mice transplanted with PTEN$^{fl/fl}$Rosa-26cre-ERT cells compared to mice transplanted with PDK1$^{fl/fl}$ PTEN$^{fl/fl}$Rosa-26cre-ERT cells. (C) Cell morphology from the bone marrow and spleen showing invasion of lymphoblasts.(D) FACS profile of ALL mouse transplanted with PDK1$^{MGK465/MGK465}$ PTEN$^{fl/fl}$Rosa-26cre-ERT cells. (E) FACS profile of AML/ALL mouse transplanted with PDK1$^{MGK465/MGK465}$PTEN$^{fl/fl}$Rosa-26cre-ERT cells.

4.5.4 PDK1 MGK465 mutation depletes ALL stem cells in PTEN null induced ALL

Our earlier data showed that PTEN null ALL primary cells isolated from mice transplanted with $PTEN^{fl/fl}$Rosa-26cre-ERT cells are able to engraft and induce ALL in secondary recipient mice. Moreover all mice injected with 1 Mio or 0.1Mio cells die within less than 30 days. We investigate the functionality of ALL cells isolated from mice transplanted with $PDK1^{MGK465/MGK465}PTEN^{fl/fl}$Rosa-26cre-ERT cells by setting up similar limiting dilution transplant assay (Fig 4.20A). We observed that when we transplant 1 mio of ALL cells isolated from mice transplant with $PDK1^{MGK465/MGK465}PTEN^{fl/fl}$Rosa-26cre-ERT cells only 2 mice died within 60 and whereas transplanting 0.1 mio or 0.01 mio cells was not able to induce leukaemia past 60 days.Our data shows that PDK1 MGK456 mutation significantly depletes ALL stem cells in PTEN deletion mediated ALL (Fig4.20B and C).



Group	Lower	Estimate	Upper
MGK465PTEN	8908464	2237898	562183
PTEN	112002	41704	15529

Figure 4.20 PDK1 MGK465 mutation depletes PTEN null ALL stem cells.

(A) Graph showing survival of mice transplanted with different number of PTEN null ALL cells compared to mice transplanted with different numbers of PDK1 MGK465 mutant PTEN null ALL cells. (B) Leukemic stem cells frequency of PTEN null ALL stem cells vs PDK1 MGK465mutant PTEN null ALL stem cells. LDA graph showing stem cells frequency of PTEN null ALL stem cells vs PDK1 MGK465 mutant PTEN null ALL stem cells.

3.6 PDK1 L155E mutation has little influence on the PTEN deletion phenotype

To investigate the role of the other 'arm' of PDK1 , i.e. PIF pocket dependent PDK1 signal transduction which mediates the phosphorylation of SGK1, RSK, PKC and S6K, we analysed the PDK1$^{L155E/L155E}$PTEN $^{fl/fl}$ Rosa-26cre-ERT transplantation experiment. While the initial engraftment levels were very comparable to other experiments (70%), we also observed in PDK1$^{L155E/L155E}$Rosa-26cre-ERT group a decrease in engraftment levels after tamoxifen treatment over time (Fig 4.21A). Interestingly, the PDK1$^{L155E/L155E}$PTEN$^{fl/fl}$Rosa-26cre-ERT group showed an increase in engraftment over time after treatment with tamoxifen similar to the what we observed in the wt or PTEN$^{fl/fl}$Rosa-26cre-ERT group (Fig 4.21C). Analysis of the peripheral blood composition revealed almost identical results that observed in the PDK1$^{MGK465/MGK465}$PTEN$^{fl/fl}$Rosa-26cre-ERT deleted group which closely resembles that of the PTEN deleted group (fig 4.21D), suggesting that the observed effects of PDK1 on loss of PTEN mediated phenotype require both arms of PDK1 signalling, i.e. the active PH domain and PIF pocket for proper AKT and SGK activation, respectively. While the control group with PDK1$^{L155/L155}$ without PTEN deletion showed a decrease of engraftment levels over time, (Fig 4.20A), the distribution of myeloid and lymphoid cells also changed over time as a decrease of Mac1/Gr1 positive myeloid cells was observed at 12 weeks while an increase of B220 B-cells was observed at 12 weeks (Fig 4.21B). To confirm the deletion of PTEN we isolated DNA from blood samples four weeks post cre-activation. We show that PTEN was completeley deleted indicated by the absent of the floxed band (Fig 3.19E). The excision of the minigene could not be

detemined using previously decribed primers P1 and P2 (Fig 2.3) hence the presense of a floxed band (Fig 4.21F).

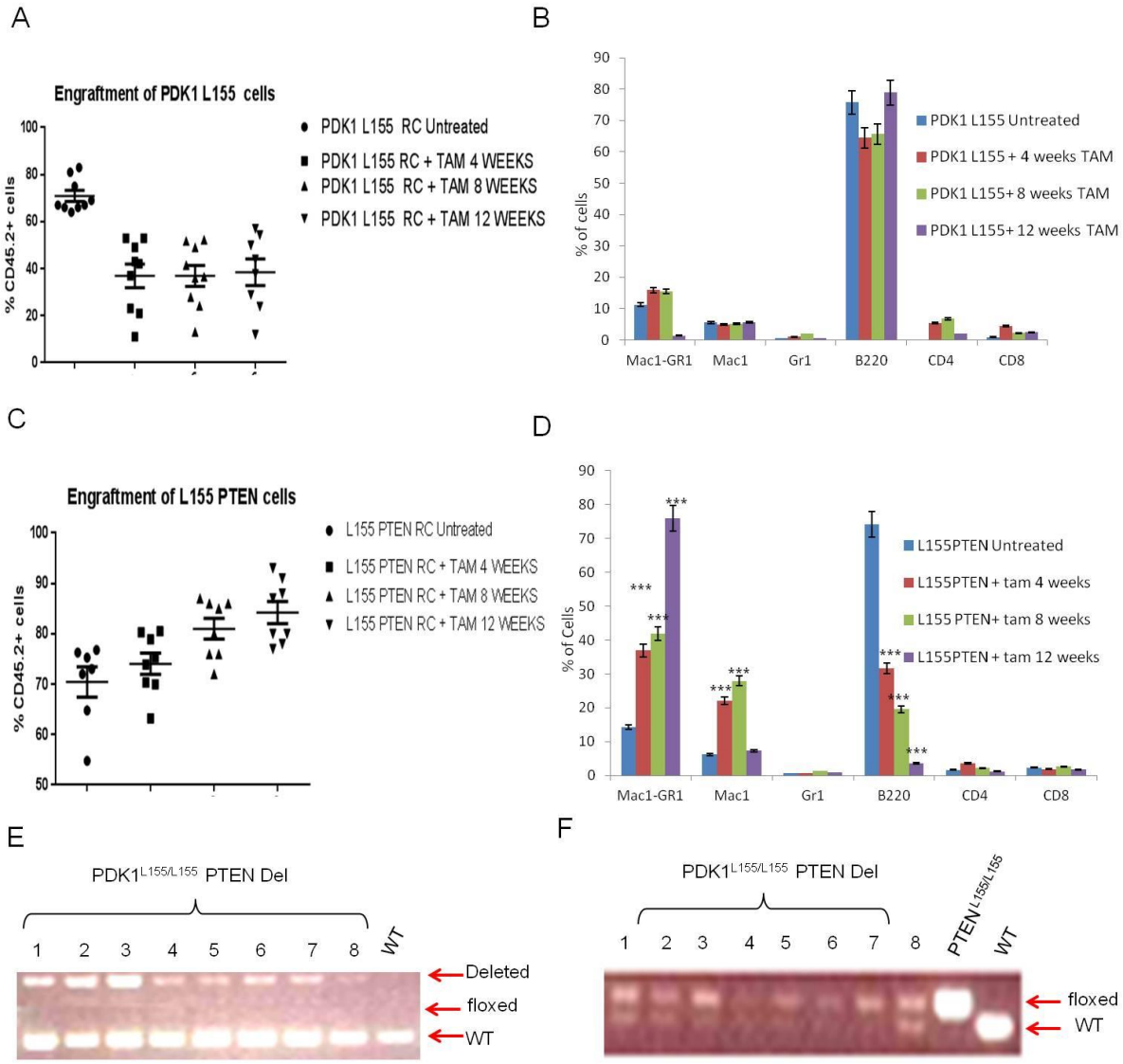


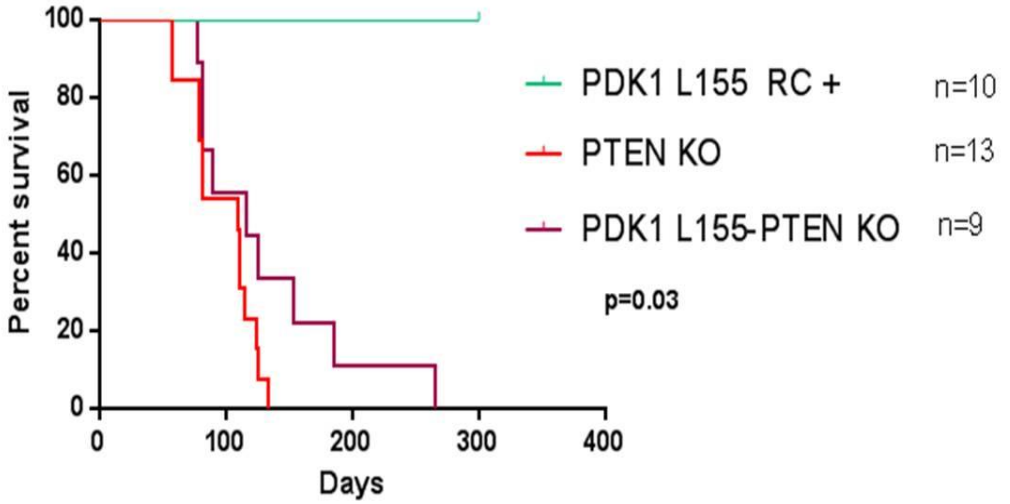
Figure 4.21 Engraftment and recostitution of PDK1 L155 mutant cells.

PDK1$^{L155/L155}$PTEN$^{fl/fl}$Rosa-26cre-ERT cells and PDK1$^{L155/L155}$Rosa-26cre-ERT cells (both CD45.2) were transplanted into SJL mice (CD 45.1). (A) and (C) The percentage of CD45.2 donor cells 4 weeks after transplantation and every 4 weeks after tamoxifen treatment is shown. (B) and (D) Immunophenotying of donor cells before and after tamoxifen with treatment is shown. Significance (* P <0.05. ** P >0.005 *** P > 0.001). (E) PCR showing complete deletion of PTEN. (F) PCR showing floxed PDK1$^{L155/L155}$ mutant allele.

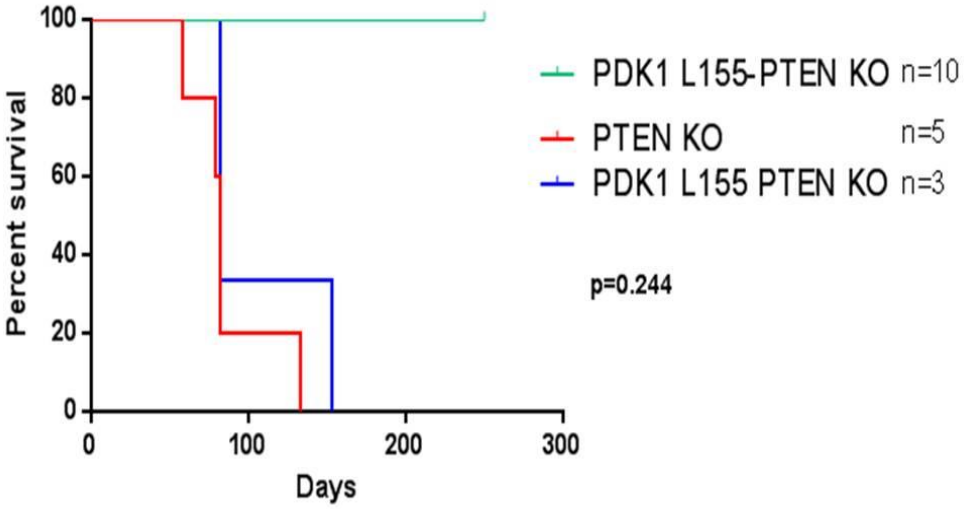
4.6.1 PDK1 L155 mutation has little influence on the PTEN mediated leukemogenesis

Mouse groups that received PDK1$^{L155E/L155E}$PTEN$^{fl/fl}$ Rosa-26cre-ERT cells succumbed to leukaemia between 70 and 270 days. All nine from this group succumbed to leukaemia and there was no significant difference when we compared the survival transplanted with PTEN$^{fl/fl}$ Rosa-26cre-ERT cells of mice that received PDK1$^{L155E/L155E}$PTEN$^{fl/fl}$ Rosa-26cre-ERT cells (Fig 4.22A). Moreover, analysis of the bone marrow, spleen, liver and thymus carried out carried out revealed that mice in this group succumbed to AML, ALL or biphenotype AML/ALL similar to what we observed previously in mice that received PTEN$^{fl/fl}$Rosa-26cre-ERT cells. Similarly, mice that came down with AML also presented with enlarged spleen and liver while those with ALL presented with a large thymic mass comparable to what we observed in mice that received PTEN$^{fl/fl}$ Rosa-26cre-ERT cell. Interestingly, when we compared the onset of AML in this group to AML in the PTEN$^{fl/fl}$Rosa-26cre-ERT group, we discovered that there was no significant difference on the latency of disease (Fig 4.22B). Three out of nine mice in this group came down with AML. Cell morphology from bone marrow and spleen revealed increased frequency of myeloid blasts (Fig 4.22C). FACS analysis revealed that the AML phenotype in this group was characterised by expansion of the double positive Mac1 and Gr1 population while depleting all other lineages (Fig 4.22D). The phenotype of cells observed in the bone marrow spleen and liver was comparable to the observed phenotype found in mice that received PTEN$^{fl/fl}$Rosa-26cre-ERT cells.

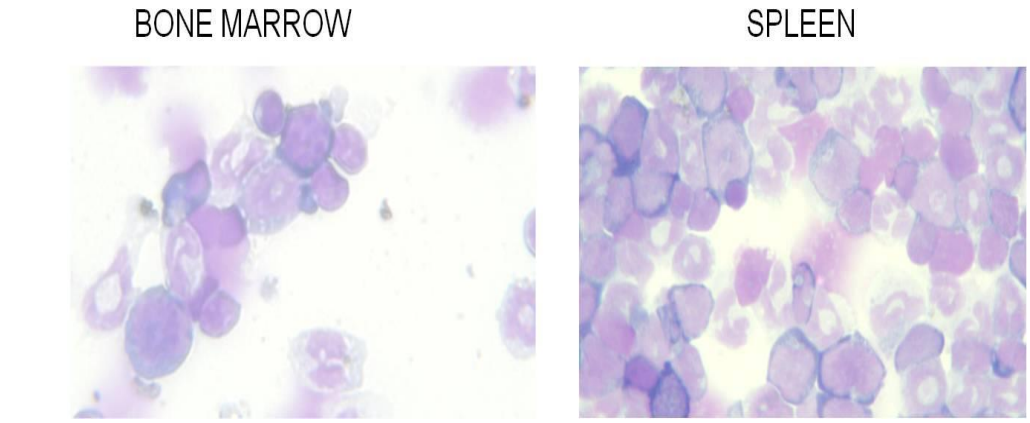
A


Percent survival
100
80
60
40
20
0
0
100
200
300
400
Days
PDK1 L155 RC +
n=10
PTEN KO
n=13
PDK1 L155-PTEN KO
n=9
p=0.03

B


Percent survival
100
80
60
40
20
0
0
100
200
300
Days
PDK1 L155-PTEN KO
n=10
PTEN KO
n=5
PDK1 L155 PTEN KO
n=3
p=0.244

C


BONE MARROW
SPLEEN

D

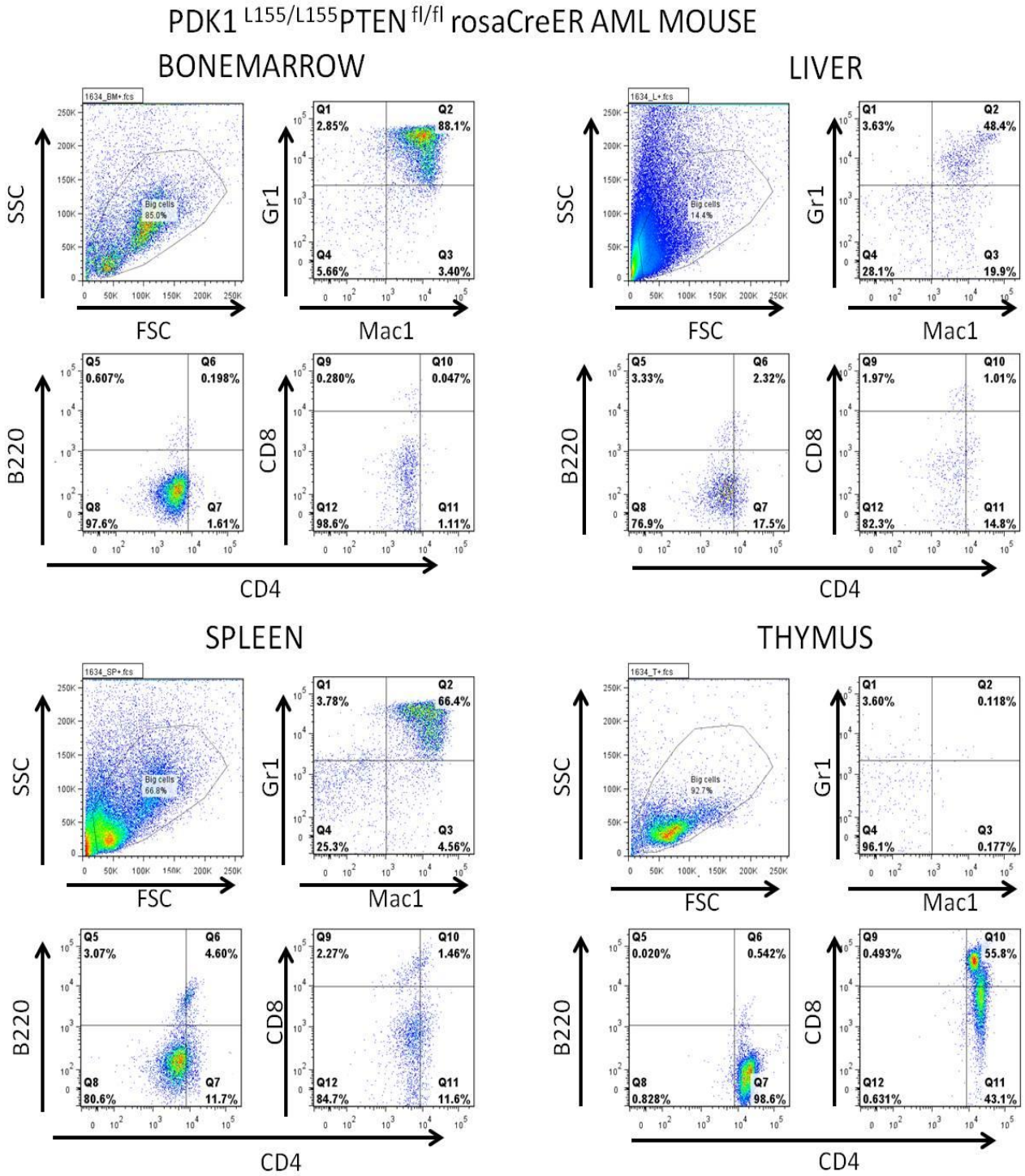


Figure 4.22 Deletion of PTEN induces leukaemia in PDK1 L155 mutant cells.

(A) Graph showing the development of leukaemia in mice transplanted with PTEN$^{fl/fl}$Rosa-26cre-ERT cells compared to mice transplanted with PDK1$^{L155/L155}$ PTEN$^{fl/fl}$ Rosa-26cre-ERT cells and mice transplanted with PDK1$^{L1555/L1555}$ cells. (B) Graph showing the development of AML in mice transplanted with PTEN$^{fl/fl}$Rosa-26cre-ERT cells compared to mice transplanted with PDK1$^{fl/fl}$ PTEN$^{fl/fl}$Rosa-26cre-ERT cells and mice transplanted with PDK1$^{L155/L155}$ cells (P value =0.244 PTEN$^{fl/fl}$Rosa-26cre-ERT vs PDK1$^{L155/L155}$PTEN$^{fl/fl}$Rosa-26cre-ERT). (C) Cell morphology from bone marrow and spleen revealed increased frequency of myeloid blasts (D)FACS profile of AML mice transplanted with PDK1$^{L155/L1555}$PTEN$^{fl/fl}$Rosa-26cre-ERT cell

4.6.2 PDK1 L155 mutation has no effect on SGK1 signalling in PTEN null AML cells

To gain further insight in the function of PDK1, we isolated bone marrow cells from mice that came down with AML from the PDK1$^{L155E/L155E}$PTEN$^{fl/fl}$Rosa-26cre-ERT group for biochemical analysis. In parallel, we also used bone marrow cells isolated from wt and PDK1$^{L155E/L155E}$Rosa-26cre-ERT group. As previously described, cells isolated from PTEN$^{fl/fl}$Rosa-26cre-ERT AML showed high levels of phosphorylated AKT Thr308 , AKT Ser473 and SGK1 Ser206 (Fig 4.23). As expected, PDK1$^{L155E/L155E}$Rosa-26cre-ERT cells were not able to phosphoprylate SGK1 Ser206 due to the defective PIF pocket. PDK1$^{L155E/L155E}$Rosa-26cre-ERT cells also could not show phosphorylation of AKT Thr308, which might be due to the fact that normal stimulation of the PI3K pathway with IGF was not induced in these cells used in this experiment. However, the wild type cells showed baseline phosphorylation of SGK1 Ser206 without sitmilation, therefore giving evidence that PDK1$^{L155E/L155E}$ Rosa-26cre-ERT cells are indeed defective in phosphorylation of SGK1 Ser206. Remarkably, PDK1$^{L155E/L155E}$Rosa-26cre-ERT cells regained phosphorylation of SGK1 upon PTEN deletion. This is consistent with our earlier findings that deletion of PTEN bypasses the normal requirement of PDK1 to activate downstream targets such as AKT and SGK1 in leukaemic cells. To futher confirm that expression of the mutant allele and excision of the minigene, DNA isolated from the bone marrow of leukaemic mice in this group was sent Transnetyx laboratories for genotyping. Their result confirmed the expression of the mutant allele (PDK1$^{L155/L155}$) and absence of the minigene (PDK1 TG) (Table 4.1). Although one of the sample showed be positive for the minigene the signal was lower than the positive control.

Table 4.1 Automated genotyping from Transnetxy

Mousenumber	Primers			
	Cre	PTEN $^{fl/fl}$	PDK1 $^{L155/L155}$	PDK1 TG
1631	+	-	++	-
1632	+	-	++	+
1633	+	-	++	-
1634	+	-	++	-
1635	+	-	++	-
1637	+	-	++	-
1638	+	-	++	UD2
Control	-	+	++	+
Control	+	-	-	-

UD2 insufficient DNA

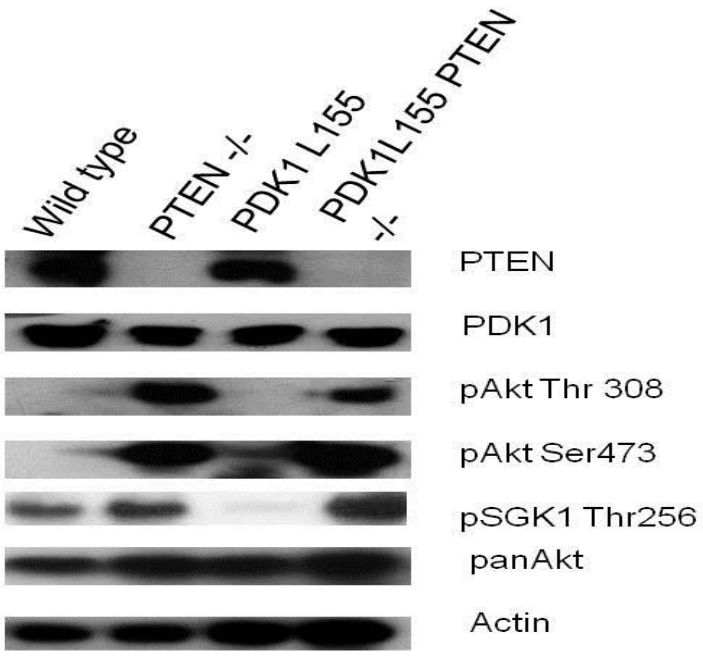


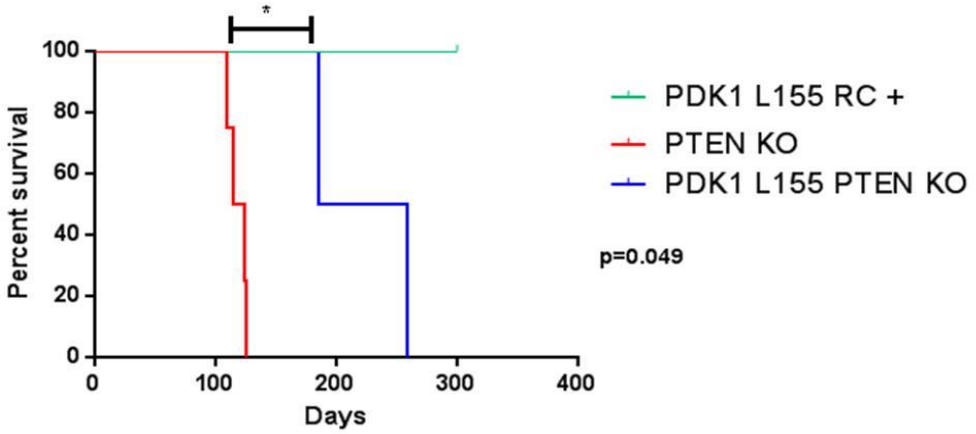
Figure 4.23 PDK1 L155 mutation has no effect on phosphorylate of SGK1 in PTEN null AML cells.

Western blot analysis of AKT Thr308, Ser473 and SGK1 Thr256 phosphorylation in PTEN$^{fl/fl}$Rosa-26cre-ERT and PDK1$^{L155/L155}$PTEN$^{fl/fl}$ Rosa-26cre-ERT bone marrow leukaemic cells.

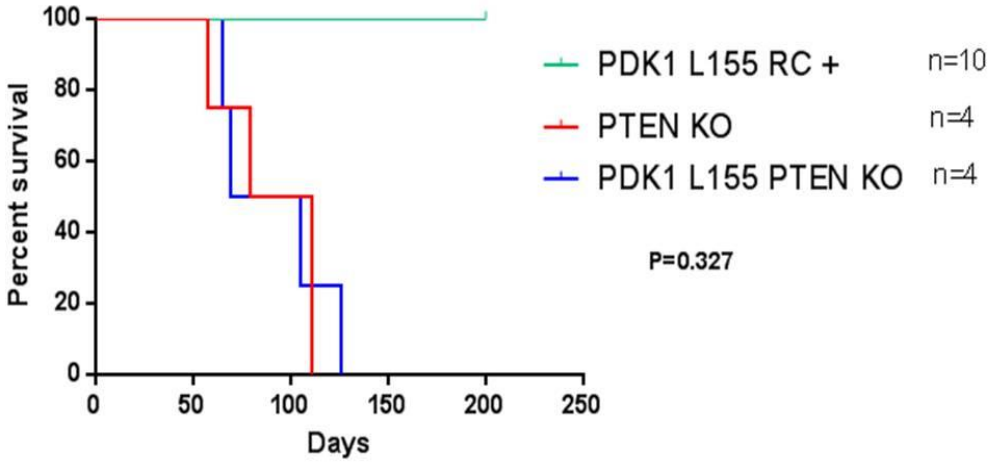
4.6.3 PDK1 L155 Mutation delayed the onset of ALL

To investigate the impact of PDK1 L155 mutation on ALL phenotype, we compared the latency of ALL in mice that received PDK1$^{L155E/L155E}$ PTEN$^{fl/fl}$Rosa-26cre-ERT cells to the latency of ALL in mice that received PTEN$^{fl/fl}$Rosa-26cre-ERT cells. Two out of the nine mice injected with PDK1$^{L155E/L155E}$PTEN$^{fl/fl}$Rosa-26cre-ERT cells came down with ALL phenotype. Interestingly, we observed that mice that received PTEN$^{fl/fl}$Rosa-26cre-ERT cells and eventually succumbed to ALL died within 120 days after PTEN deletion, whereas mice that received PDK1$^{L155E/L155E}$PTEN$^{fl/fl}$Rosa-26cre-ERT cells succumb to ALL after 220 days post PTEN deletion. We found the difference in latency of ALL between the two groups was significant (Fig 4.24A). FACS analysis of the bone marrow, spleen, liver and thymus revealed infiltration of these organs with T-lymphocytes that are double positive for CD4 and CD8 markers comparable to what we observed in the PTEN$^{fl/fl}$Rosa-26cre-ERT group. Cell morphology from the bone marrow and spleen revealed an increase in lymphoblast (Fig 4.24C). Similar to our earlier observation in mice that received PTEN$^{fl/fl}$Rosa-26cre-ERT cells and PDK1$^{MGK465/MGK465}$PTEN$^{fl/fl}$Rosa-26cre-ERT cells, a fraction of mice that received PDK1$^{L155E/L155E}$PTEN$^{fl/fl}$Rosa-26cre-ERT cells came down with a phenotype we described as AML/ALL biphenotype. Four mice in this group came down with this bi-phenotype; however when we compare the latency of disease in these mice compared to mice that came down with the same phenotype of disease in the PTEN$^{fl/fl}$Rosa-26cre-ERT group, the similar latency was observed between two groups (Fig 4.24B). The AML/ALL phenotype in these mice showed expansion of myeloid cells in the bone marrow comparable to AML phenotype whereas the liver showed expansion of T-lymphocytes comparable to ALL phenotype (Fig 4.24D).

A

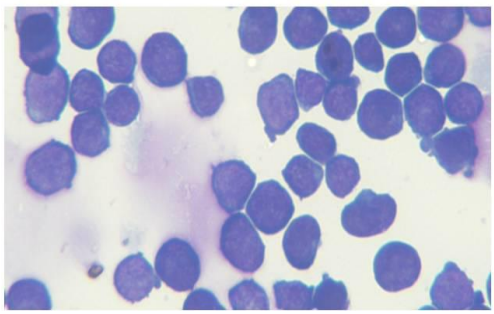


B

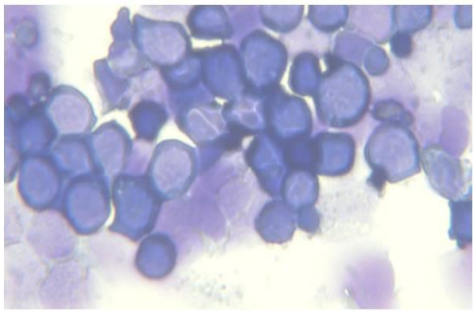


C

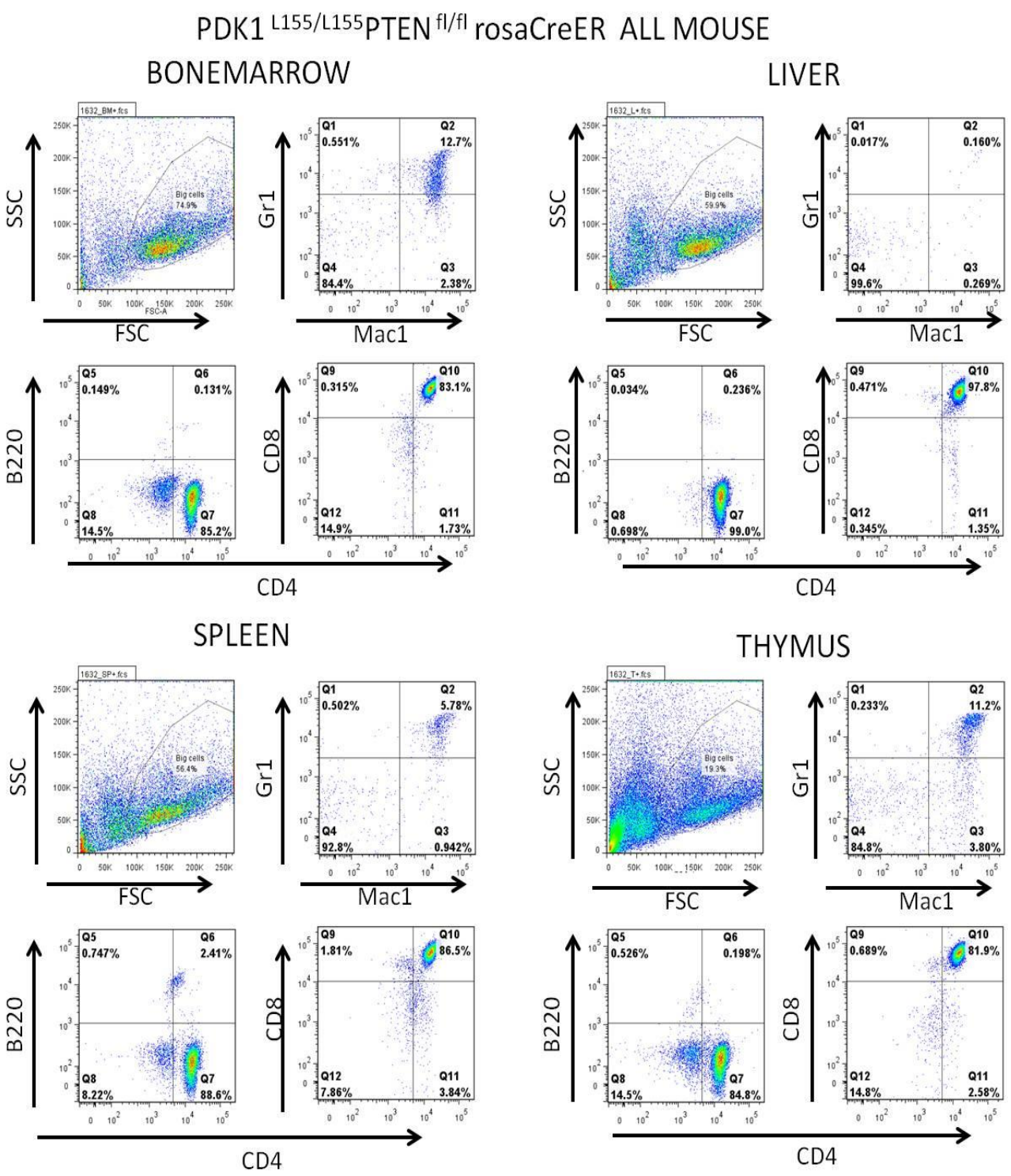
BONE MARROW



SPLEEN



D


PDK1 L155/L155PTEN fl/fl rosaCreER ALL MOUSE
BONEMARROW
LIVER
SPLEEN
THYMUS
SSC
FSC
Gr1
Mac1
B220
CD8
CD4
Big cells 74.9%
Q1 0.551%
Q2 12.7%
Q4 84.4%
Q3 2.38%
Q5 0.149%
Q6 0.131%
Q8 14.5%
Q7 85.2%
Q9 0.315%
Q10 83.1%
Q12 14.9%
Q11 1.73%
Big cells 59.9%
Q1 0.017%
Q2 0.160%
Q4 99.6%
Q3 0.269%
Q5 0.034%
Q6 0.236%
Q8 0.698%
Q7 99.0%
Q9 0.471%
Q10 97.8%
Q12 0.345%
Q11 1.35%
Big cells 56.4%
Q1 0.502%
Q2 5.78%
Q4 92.8%
Q3 0.942%
Q5 0.747%
Q6 2.41%
Q8 8.22%
Q7 88.6%
Q9 1.81%
Q10 86.5%
Q12 7.86%
Q11 3.84%
Big cells 19.3%
Q1 0.233%
Q2 11.2%
Q4 84.8%
Q3 3.80%
Q5 0.526%
Q6 0.198%
Q8 14.5%
Q7 84.8%
Q9 0.689%
Q10 81.9%
Q12 14.8%
Q11 2.58%

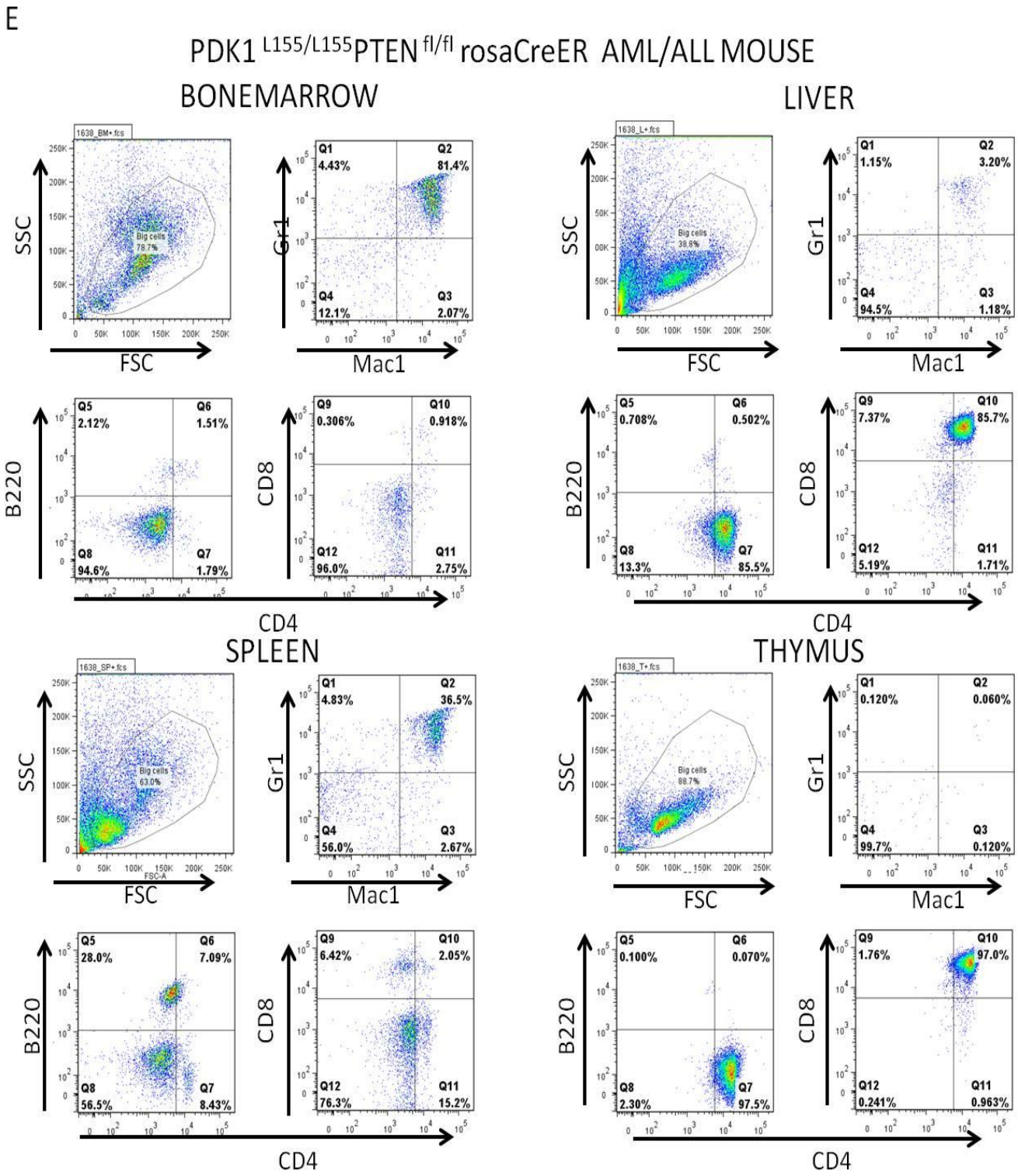
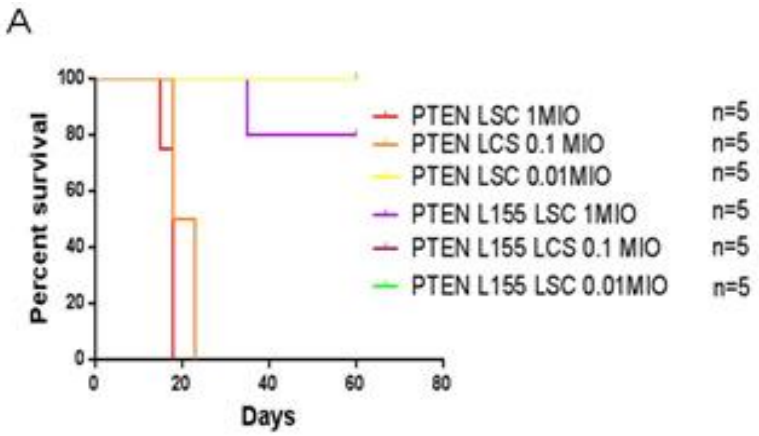


Figure 4.24 PTEN deletion induced ALL and AML/ALL phenotypes in PDK1 L155 mutant.

(A) Graph showing the development of ALL in mice transplanted with PTEN$^{fl/fl}$Rosa-26cre-ERT cells compared to mice transplanted with PDK1$^{L155/L155}$ PTEN$^{fl/fl}$Rosa-26cre-ERT cells. (B) Graph showing the development of AML/ALL in mice transplanted with PTEN$^{fl/fl}$Rosa-26cre-ERT cells compared to mice transplanted with PDK1$^{fl/fl}$ PTEN$^{fl/fl}$Rosa-26cre-ERT cells. (C) Bone marrow and spleen cell morphology showing increased lymphoblasts. (D) FACS profile of ALL mouse transplanted with PDK1$^{L155/L155}$PTEN$^{fl/fl}$Rosa-26cre-ERT cells. (E) FACS profile of AML/ALL mouse transplanted with PDK1$^{L155/L155}$ PTEN$^{fl/fl}$Rosa-26cre-ERT cells.

4.6.4 PDK1 L155 mutation depletes ALL stem cells in PTEN null induced ALL

To test the functionally of ALL stem cells isolated from mice transplanted with $PDK1^{L155/L155}PTEN^{fl/fl}$Rosa-26cre-ERT cells, we set up an limiting dilution transplant assay. We transplant 1 mio, 0.1 mio and 0.01 mio cells into lethally irradiated mice (n=5) together with 0.2 mio wild type cells for rescue. We observed that when we transplant 1 mio of ALL cells isolated from mice transplant with $PDK1^{L155/L155}PTEN^{fl/fl}$Rosa-26cre-ERT cells only one mouse died within 60 and whereas transplanting 0.1 mio or 0.01 mio cells was not able to induce leukaemia past 60 days (Fig 4.25A). Our data shows that PDK1 L155 mutation depletes ALL stem cells in PTEN deletion mediated ALL (Fig 3.23B and C).



B

Group	Lower	Estimate	Upper
L155PTEN	35590717	5039009	713434
PTEN	112002	41704	15529

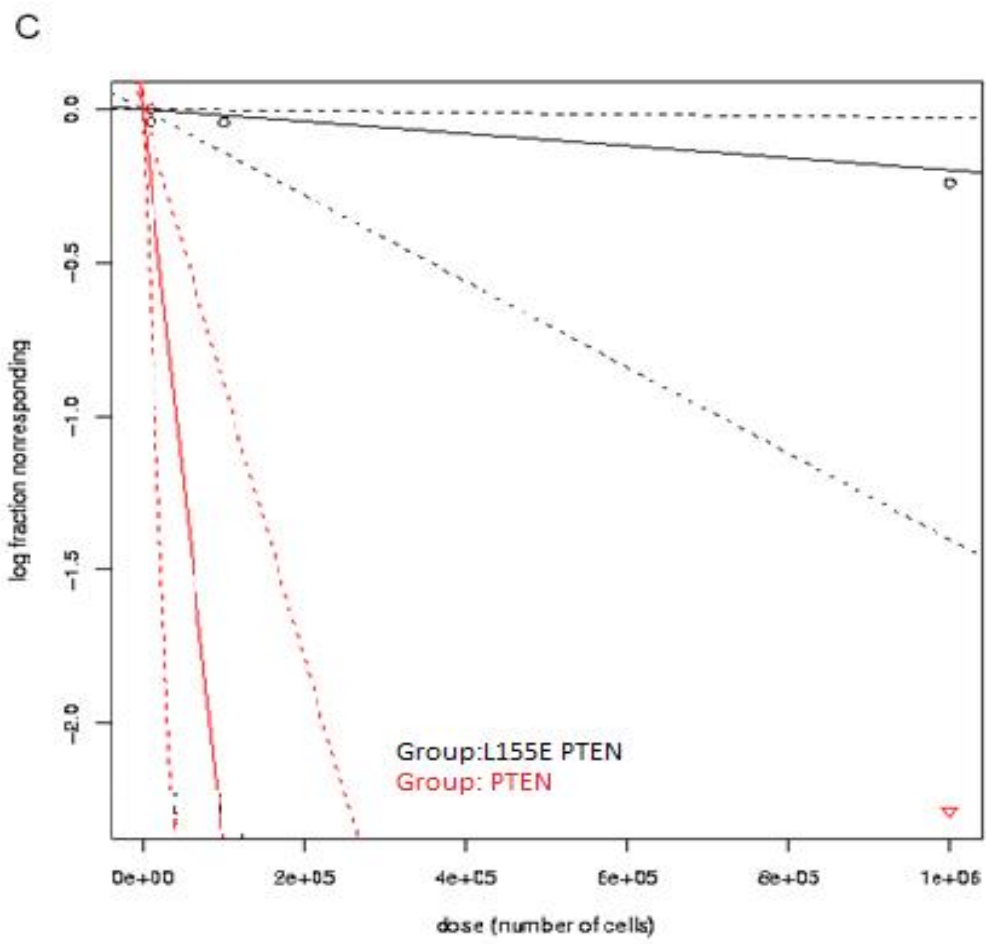


Figure 3.23 PDK1 L155 mutation depletes PTEN null ALL stem cells.

(A) Graph showing survival of mice transplanted with different number of PTEN null ALL cells compared to mice transplanted with different numbers of PDK1 L155 mutant PTEN null ALL cells. (B) Leukaemic stem cells frequency of PTEN null ALL stem cells vs PDK1 MGK465 mutant PTEN null ALL stem cells. (C) LDA graph showing stem cells frequency of PTEN null ALL stem cells vs PDK1 MGK465 mutant PTEN null ALL stem cells.

4.7 Simultaneous deletion of PTEN and PDK1 using the Vav-cre

We have established that PTEN deletion in haematopoietic cells using a Rosa-26cre-ERT system in a transplantation setting causes leukaemia. We further showed that deletion PTEN and PDK1 using the Rosa-26cre-ERT system has an impact on the latency of leukaemia using transplant studies. To support and strengthen our finding, we used another Cre mouse model which utilizes the Vav-cre sytem to initiate the deletion of PTEN and PDK1 only in the haematopoietic cells. To this end, we generated $PTEN^{fl/fl}$ Vav-cre, $PDK1^{fl/fl}$ Vav-cre and compound knockout $PTEN^{fl/fl}PDK1^{fl/fl}$ Vav-cre. Single $PTEN^{fl/fl}$ Vav-cre mice were viable and developed to adult mice. Likewise $PDK1^{fl/fl}$ Vav-cre mice were viable and developed to adult mice. Although the compound knockout $PTEN^{fl/fl}PDK1^{fl/fl}$ Vav-cre mice were viable, a few of these mice were born with abnormalities such as tiny body size and deformed head. More importantly, the breeding of compound knockout mice was very challenging and there seemed to be a negative selection against the $PTEN^{fl/fl}PDK1^{fl/fl}$ Vav-cre genotype. We produced more than 200 pups and we were only able to produce 6 mice with desired genotype and all of them were females. In addition, these females were not able to breed due to early onset of leukaemia which was unexpected if compared to the onset of leukaemia in $PTEN^{fl/fl}PDK1^{fl/fl}$ Rosa-26cre-ERT after tamoxifen treatment. We tried different breeding strategies to maximise the probability of getting more $PTEN^{fl/fl}PDK1^{fl/fl}$ Vav-cre. Surprisingly, $PTEN^{fl/wt}PDK1^{fl/fl}$ Vav-cre also succumbed to illness making it much more difficult to maintain and expand this mouse line. However mice with this genotype

were not analysed so I could not confirm leukaemia. To investigate the underlying cause of illness, we bleed PTEN$^{fl/fl}$PDK1$^{fl/fl}$ Vav-cre (n=3), PTEN$^{fl/fl}$Vav cre (n=3), PDK$^{fl/fl}$ Vav-cre and PTEN$^{fl/fl}$ (n=3). Similar to our earlier finding, deletion of PTEN resulted in positive selection towards myeloid lineage and depletion of B-cells in both single PTEN knock-out and compound PTEN/PDK1 knockout (Fig 4.26). Surprisingly, we discovered that PDK1$^{fl/fl}$Vav cre showed comparable result to PTEN$^{fl/fl}$ Vav-cre.

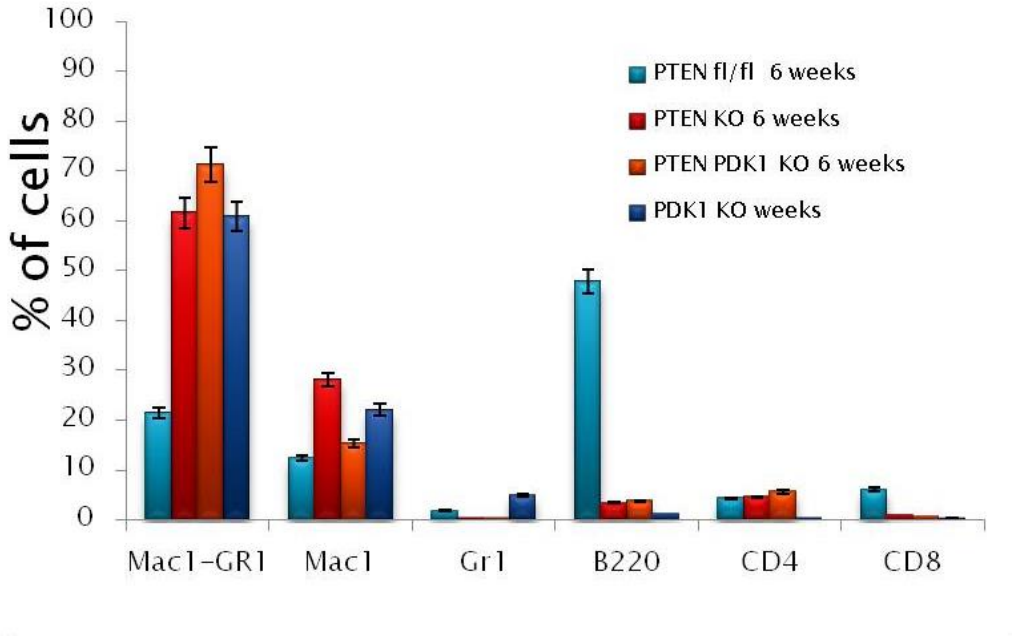


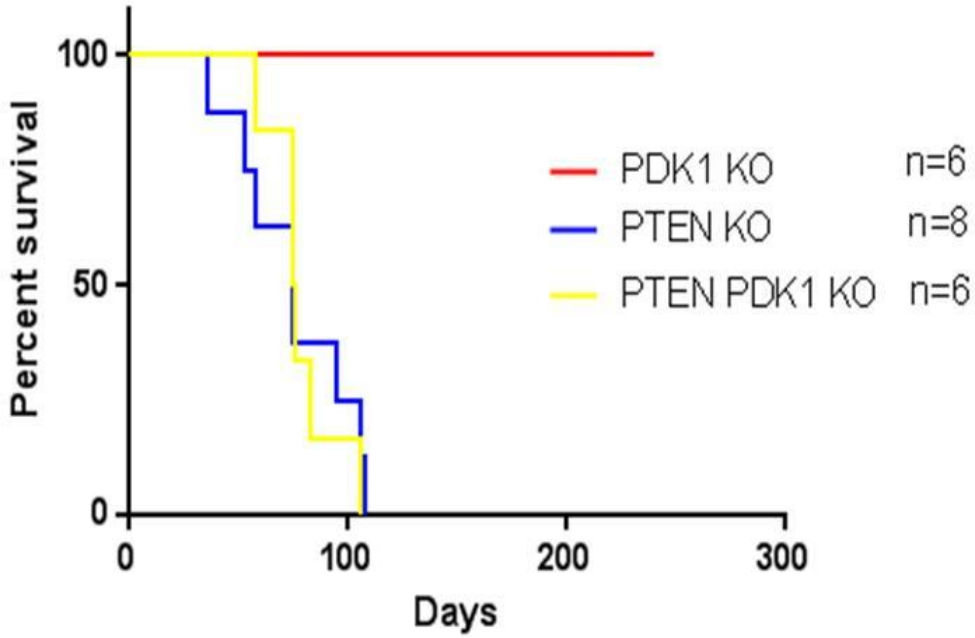
Figure 4.26 Impairment of haematopoiesis in PTEN$^{fl/fl}$ Vav-cre, PDK1$^{fl/fl}$ Vav-cre and compound PTEN$^{fl/fl}$ PDK1$^{fl/fl}$ Vav-cre mice.

PTEN$^{fl/fl}$ (n=3), PTEN KO (n=3), PTEN PDK1 KO and PDK1 KO(n=3). Immunophenotying of peripheral blood expansion of the myeloid lineage while depleting B cell lineage.

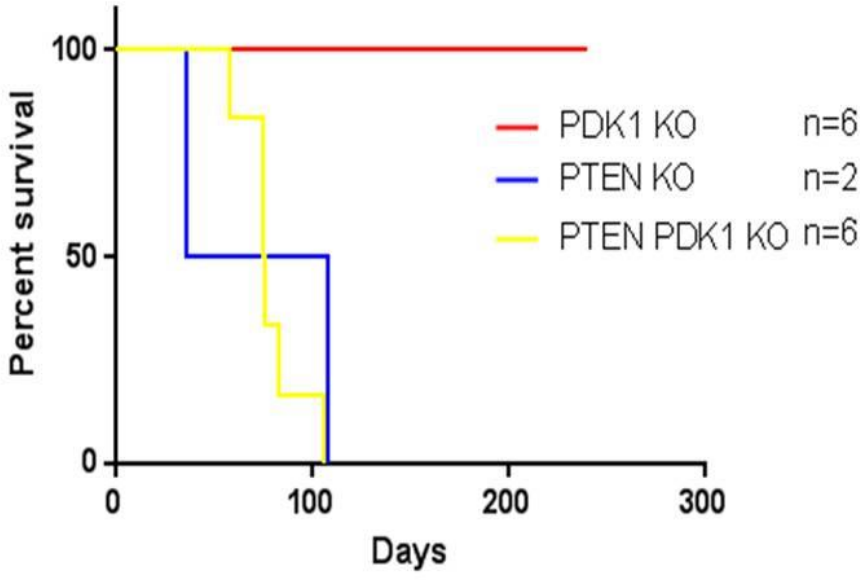
We monitored the PTEN$^{fl/fl}$PDK1$^{fl/fl}$ Vav-cre, PTEN$^{fl/fl}$ Vav-cre, PDK1$^{fl/fl}$ Vav-cre mice until they succumbed to leukaemia. We analysed eight PTEN$^{fl/fl}$ Vav-cre mice and we show that PTEN deletion resulted in leukaemia between 40 and 110 days comparable to our earlier findings using PTEN$^{fl/fl}$ Rosa-26cre-ERT. Surprisingly PTEN$^{fl/fl}$PDK1$^{fl/fl}$ Vav-cre mice succumbed to leukaemia between 50

and 110 days which is early compared to our findings using $PTEN^{fl/fl}PDK1^{fl/fl}$Rosa-26cre-ERT (Fig 4.27A). FACS analysis revealed that some of the mice came down with AML. Two out of eight $PTEN^{fl/fl}$ Vav-cre mice come down with AML whereas all six out of six $PTEN^{fl/fl}PDK1^{fl/fl}$ Vav-cre came with ALL (Fig 4.27B). The bone marrow of AML mice showed almost a complete infiltration of the bone marrow with myeloid cells and in most cases more than 70% the cells were positive for both Mac1 and Gr1 suggesting a shift in the turnover of myeloid cells (Fig 4.27C). The spleen and liver of AML mice also showed increased proportion of myeloid cells with a majority of the cells again positive for both Mac1 and Gr1. The proportion of lymphocytes was greatly reduced in the bone marrow, spleen and liver. Myeloid cells were not present in the thymus in any of the mice that came down with AML. Interestingly, while the FACS profile of AML mice was similar in $PTEN^{fl/fl}$ Vav-cre mice and $PTEN^{fl/fl}PDK1^{fl/fl}$ Vav-cre mice, we observed that myeloid cells infiltrated into the thymus of some of the $PTEN^{fl/fl}PDK1^{fl/fl}$ Vav-cre mice (Fig 4.27D).

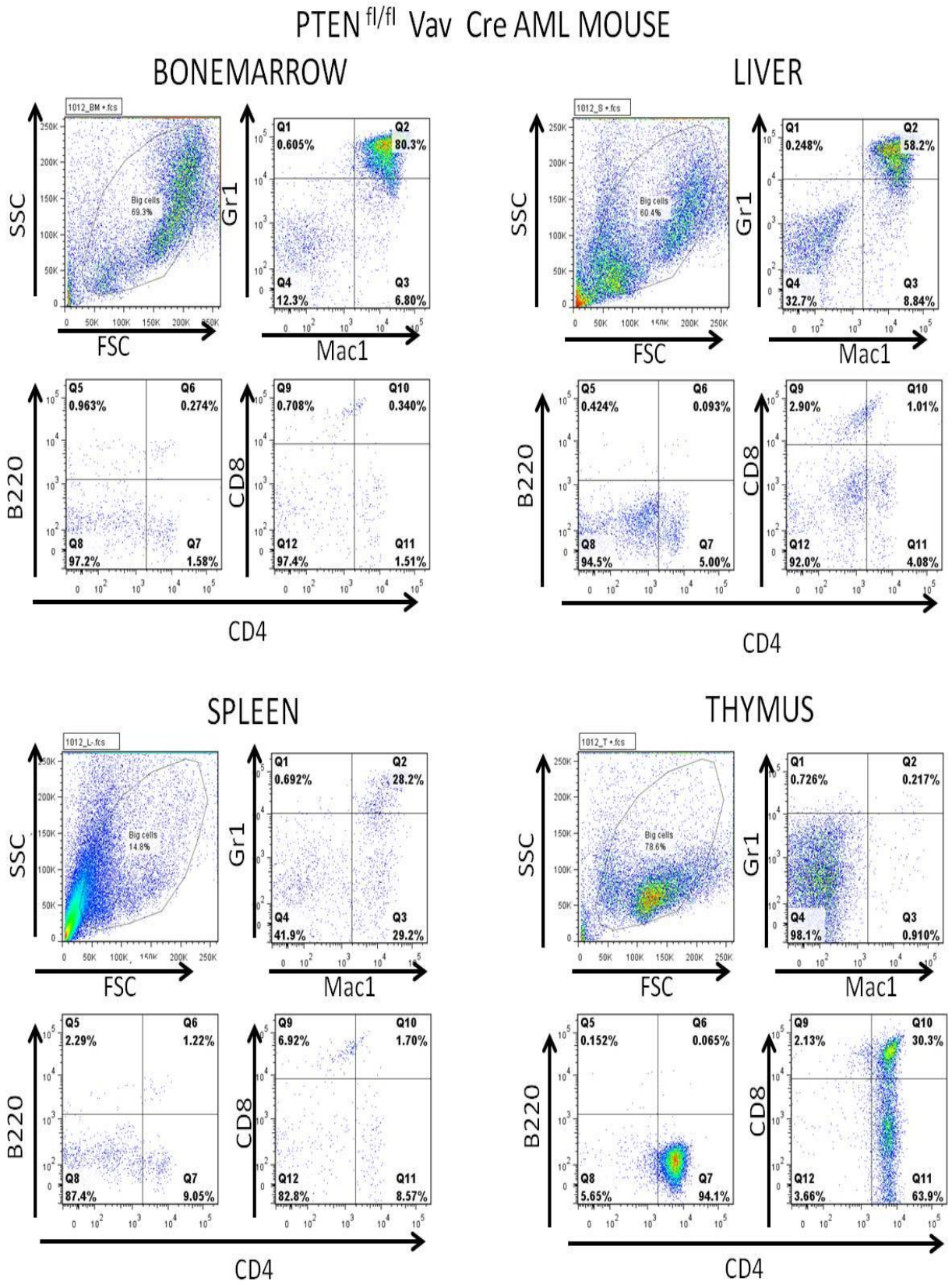
A


100
50
0
Percent survival
0
100
200
300
Days
PDK1 KO n=6
PTEN KO n=8
PTEN PDK1 KO n=6

B


100
50
0
Percent survival
0
100
200
300
Days
PDK1 KO n=6
PTEN KO n=2
PTEN PDK1 KO n=6

C


PTEN fl/fl Vav Cre AML MOUSE
BONEMARROW
LIVER
SPLEEN
THYMUS
SSC
FSC
Gr1
Mac1
B220
CD8
CD4
Big cells 69.3%
Q1 0.605%
Q2 80.3%
Q4 12.3%
Q3 6.80%
Q5 0.963%
Q6 0.274%
Q8 97.2%
Q7 1.58%
Q9 0.708%
Q10 0.340%
Q12 97.4%
Q11 1.51%
Big cells 60.4%
Q1 0.248%
Q2 58.2%
Q4 32.7%
Q3 8.84%
Q5 0.424%
Q6 0.093%
Q8 94.5%
Q7 5.00%
Q9 2.90%
Q10 1.01%
Q12 92.0%
Q11 4.08%
Big cells 14.8%
Q1 0.692%
Q2 28.2%
Q4 41.9%
Q3 29.2%
Q5 2.29%
Q6 1.22%
Q8 87.4%
Q7 9.05%
Q9 6.92%
Q10 1.70%
Q12 82.8%
Q11 8.57%
Big cells 78.6%
Q1 0.726%
Q2 0.217%
Q4 98.1%
Q3 0.910%
Q5 0.152%
Q6 0.065%
Q8 5.65%
Q7 94.1%
Q9 2.13%
Q10 30.3%
Q12 3.66%
Q11 63.9%

D

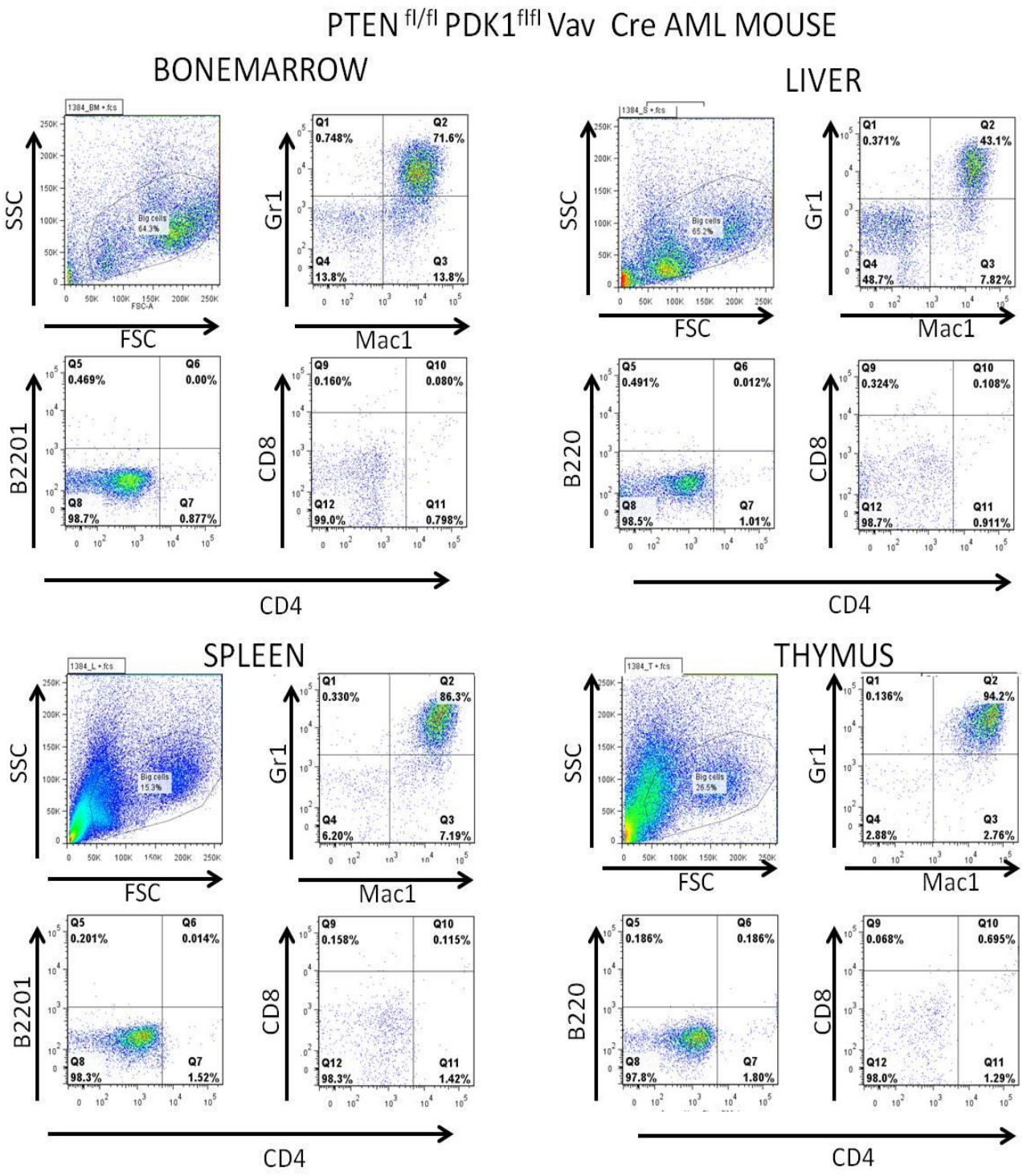
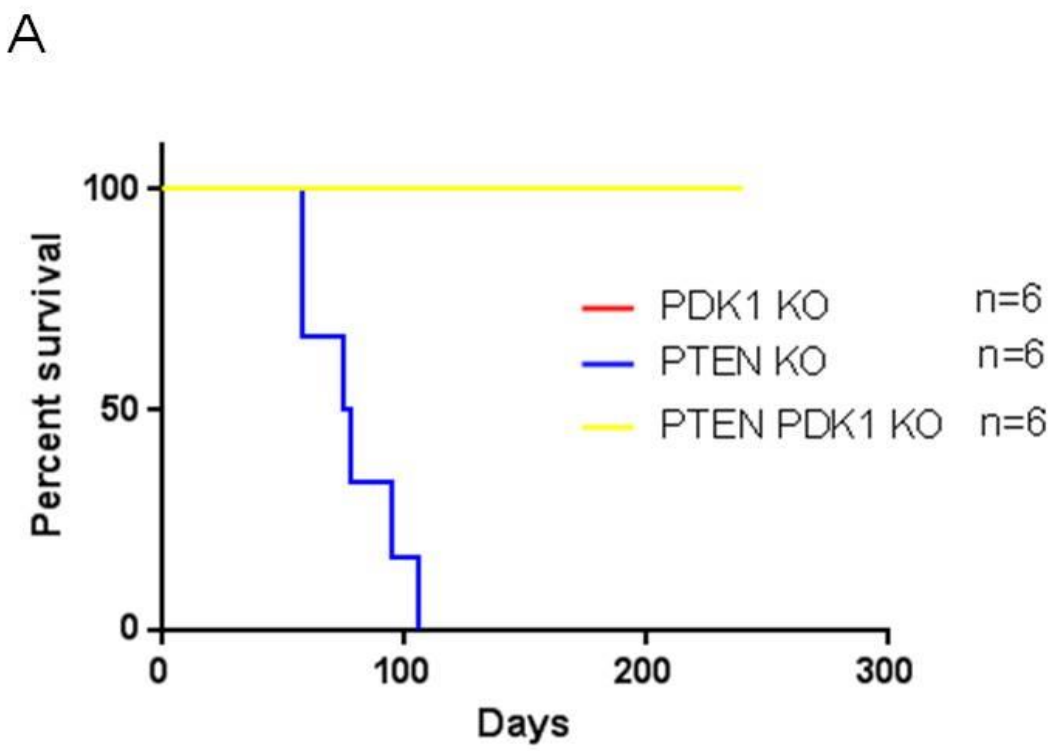


Figure 4.27. Development of leukaemia in PTENfl/fl Vav-cre and PTENfl/fl PDK1fl/fl Vav-cre mice.

(A) Graph showing the development of leukaemia in PTEN$^{fl/fl}$ Vav-cre mice compared to PTEN$^{fl/fl}$PDK1$^{fl/fl}$ Vav-mice. (B) Graph showing the development of AML in PTEN$^{fl/fl}$ Vav-cre mice compared to PTEN$^{fl/fl}$PDK1$^{fl/fl}$ Vav-mice. (C) FACS profile of AML in PTEN$^{fl/fl}$PDK1$^{fl/fl}$Vav cre mouse. (D) FACS profile of ALL in PTEN$^{fl/fl}$ Vav-cre mouse.

Similar to our previous finding where we transplanted PTEN$^{fl/fl}$Rosa-26cre-ERT cells, we observed that PTEN$^{fl/fl}$ Vav-cre mice came down either with AML or T-ALL. Moreover, again similar to the earlier Ros26Cre data, none of the PTEN$^{fl/fl}$PDK1$^{fl/fl}$ Vav-cre mice only came down with ALL (Fig 4.28A). Together, these results support that finding that PDK1 might be important in loss of PTEN mediated T-ALL. FACS analysis of the bone marrow, spleen, and liver of ALL mice showed infiltration of the bone marrow with lymphoid cells and in all cases, more than 50% the cells were positive for both CD4 and CD 8 suggesting a shift in the turnover of lymphoid cells (Fig 4.28B).



B

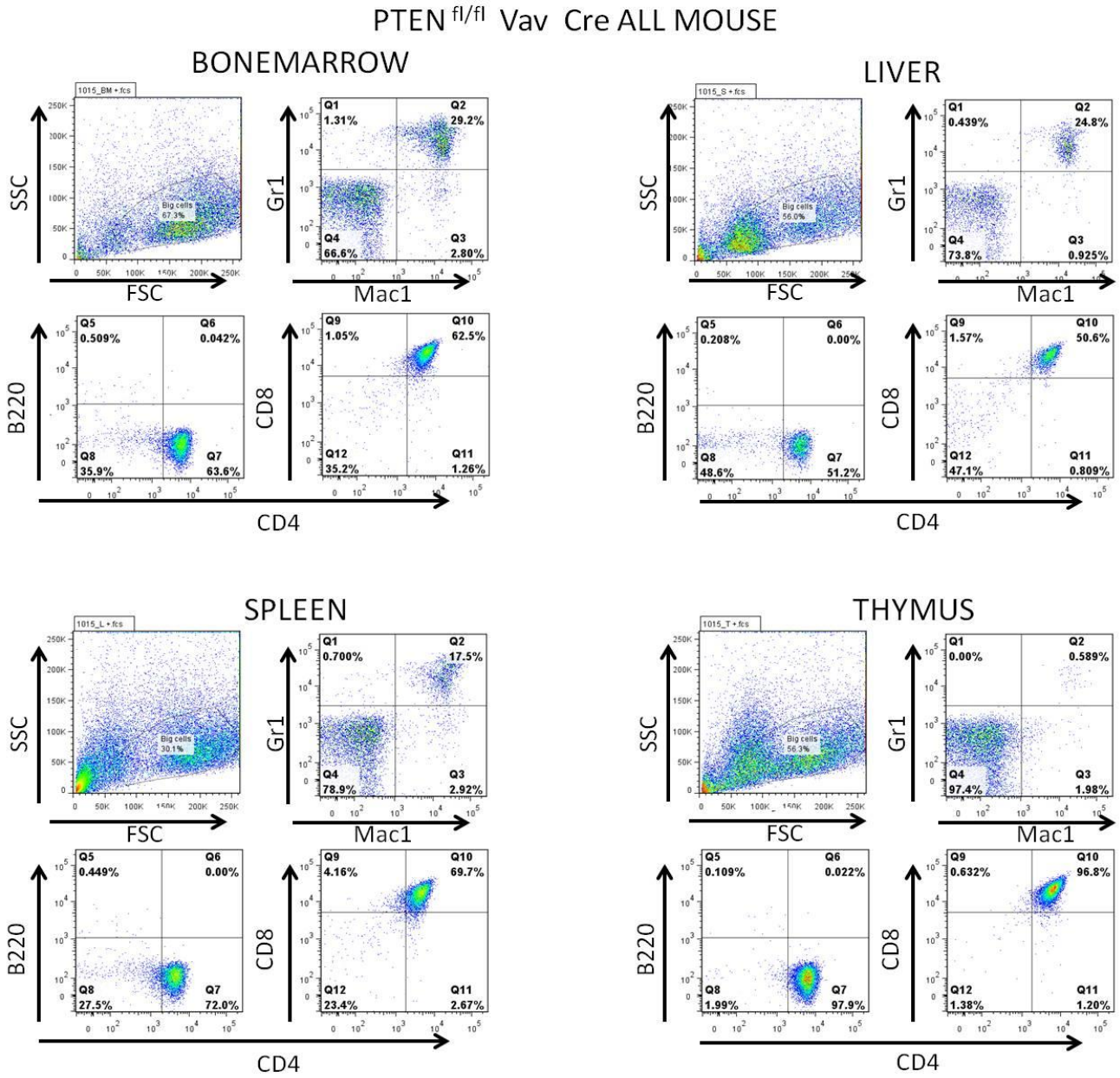


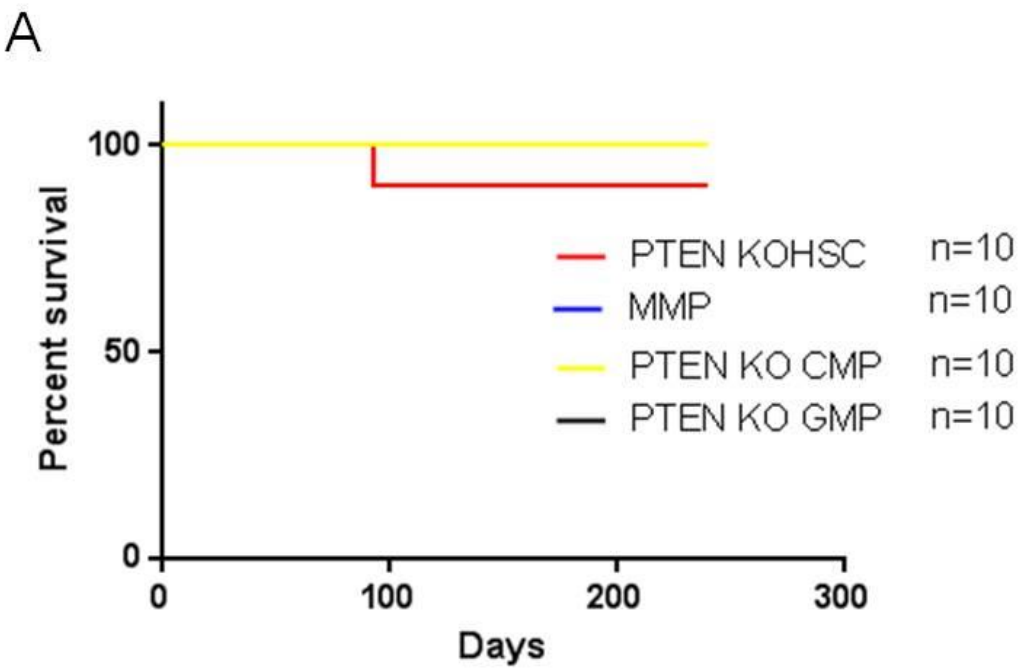
Figure 4.28 Development of ALL in PTEN$^{fl/fl}$ Vav-cre mice.

(A) Graph showing survival of mice that succumbed to ALL FACS profile of AML in PTEN$^{fl/fl}$ Vav-cre mouse. (B) FACS profile of ALL in PTEN$^{fl/fl}$ Vav-cre mouse.

4.8 Loss of PTEN induced leukaemia may have arisen from the HSC compartment

We have demonstrated that deletion of PTEN in haematopoietic cells led to myeloid proliferation that eventually developed to AML or ALL. To investigate the origin of the leukaemia in PTEN deleted in haematopoietic cells, we set up transplant using different fractions of haematopoietic cells. We administered tamoxifen (120 µl per day for five days) to induce cre activation in $PTEN^{fl/fl}$Rosa-26cre-ERT (CD45.2 n=10). Next we isolated bone marrow cells post treatment and pooled together. We sort LT-HSC ($Lin^{-/lo}Sca1^{+}c\text{-}Kit^{+}CD150^{+}CD48^{-}$), MPP ($Lin^{-/lo}Sca1^{+}c\text{-}Kit^{+}CD150^{-}CD48^{+}$), CMP ($Lin^{-/lo}Sca1^{-}c\text{-}Kit^{+}CD34^{+}CD16/32^{lo}$) and GMP ($Lin^{-/lo}Sca1^{-}c\text{-}Kit^{+}CD34^{+}CD16/32^{hi}$) from the pooled cells. We transplanted $5x10^{2}$ HSCs ($Lin^{-/lo}Sca1^{+}c\text{-}Kit^{+}CD150^{+}CD48^{-}$), $1x10^{4}$ MPPs ($Lin^{-/lo}Sca1^{+}c\text{-}Kit^{+}CD150^{-}CD48^{+}$) $1x10^{4}$ CMPs ($Lin^{-/lo}Sca1^{-}c\text{-}Kit^{+}CD34^{+}CD16/32^{lo}$) and $1x10^{4}$ GMPs ($Lin^{-/lo}Sca1^{-}c\text{-}Kit^{+}CD34^{+}CD16/32^{hi}$) together with $2X10^{5}$ wild type cells (CD45.1) into lethally irradiated mice (CD45.1). We observed that only 1 out of 10 mice transplanted with HSCs came down with ALL (Fig 4.29A). None of the mice transplanted with MPPs, CMPs or GMPs came down with leukaemia. We sacrificed the mice at 20 weeks after transplant and discovered that our donor cells were no longer detectable in the bone marrow of mice transplanted with MPPs, CMPs and GMPs (Fig 4.29B). Minimal percentage of donor cells was detected in some of the mice that received HSCs. These data suggest that deletion of PTEN affects the self-renewal of HSCs therefore resulting in stem cell depletion. The origin of leukaemia still remains to be elucidated. However, we suspect the leukaemia might have acquired secondary mutations that inactivate the senescence response of the few HSCs that remained. Yilmaz and

colleagues performed similar experiment, and they demonstrated that when they transplanted 15 Flk-2^{-}Sca-1^{+}Lin^{-}c-Kit^{+}CD48^{-} (HSCs) into irradiated mice none of the mice came down with leukaemia. They further argued that PTEN deficient HSC were able to engraft and initially gave multi-lineage reconstitution, but by eight weeks after transplant, none of the mice retained multi-lineage reconstitution. Moreover, the level of reconstitution and donor chimaerism continuously declined (Yilmaz et al., 2006b).



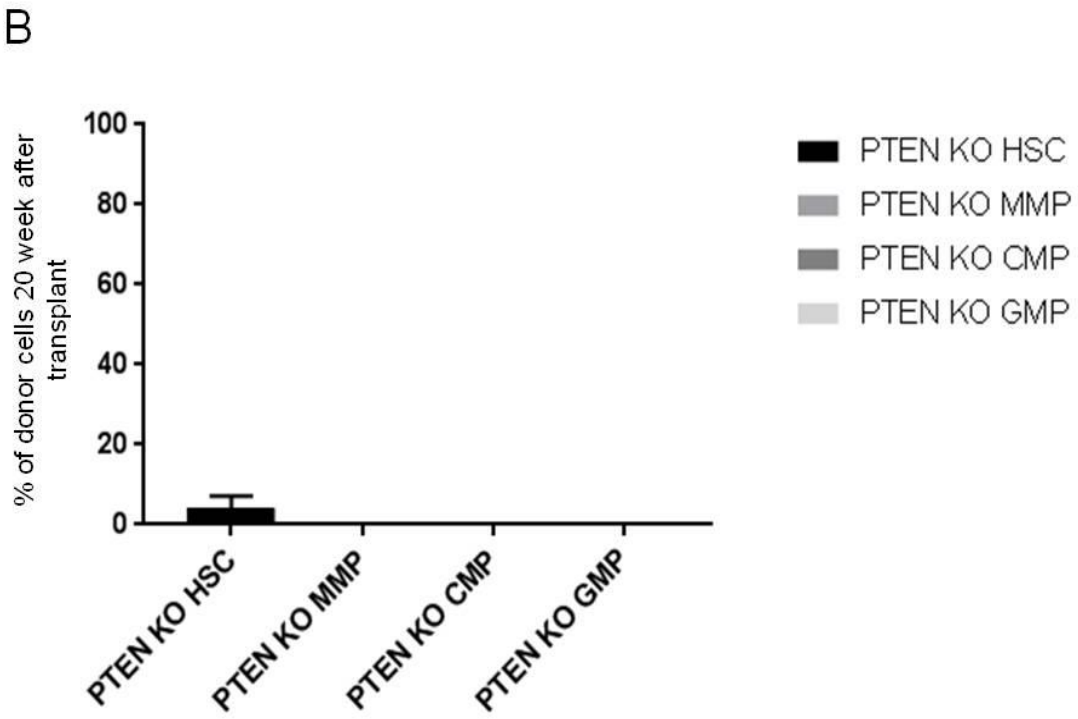


Figure 4.29 Origin of the PTEN deletion induced leukaemia.

(A) Survival of mice after transplant with different fractions of PTEN deleted haematopoietic cells. (B) Percentage of donor cells (CD45.2) 20 weeks after transplant.

Discussion

We have generated single and compound conditional knockout/KI mouse lines with PTEN and PDK1 as well as PDK1 mutants (PDK1 L155E MG with disrupted PDK1-PIF pocket domain and PDK1 K465E a defective PDK1-PH domain) to investigate the role of PDK1 in PTEN haematological neoplasms. Understanding the consequences of loss of PTEN function at a molecular level and its contribution to leukaemia development are critical for cancer biology in general and for development of rational therapeutics in particular. While several studies have reported that PTEN is one of the most frequently mutated tumour suppressor genes in human cancers (Guo et al., 2011, Fragoso and Barata, 2014); its mutation frequency in haematopoietic malignancy is relatively low when compared to other cancers (Denning et al., 2007, Chalhoub and Baker, 2009, Barata, 2011). Strikingly, PTEN mutations vary greatly in different haematological malignancies with around 2% of AML cases (Zeisig et al., 2012) and 8-63% of paediatric T-ALL (Gutierrez et al., 2009, Medyouf et al., 2010). However, our mouse model shows that AML is more frequent than previously reported in PTEN deletion induced leukaemia. Moreover, earlier studies using PTEN mouse models have clearly demonstrated a functional role for PTEN in normal and malignant haematopoiesis (Yilmaz et al., 2006b, Zhang et al., 2006). Yilmaz and colleagues demonstrated that 6 to 8 weeks old PTEN $^{fl/fl}$ Mx-1-cre mice developed a myeloproliferative disease that progressed to acute leukaemia development within 4 to 6 weeks and exhausted the normal haematopoietic stem cell pool after activation of cre with pIpC treatment to delete of PTEN.

Similarly, we showed that PTEN deletion seems to preferentially favour the proliferation of myeloid cells over lymphoid cells. It is tempting to speculate that the increased proliferation of myeloid cells may have led to development of AML. Interestingly, while we could not observe any significant changes in the proportion of T cells in the peripheral blood, some of the mice developed T-ALL, despite myeloid cell expansion in the peripheral blood suggesting that there could be an underlying expansion of T lymphocytes in other organs. However, it is worth noting that some of these mice came down with both AML/ALL. The AML/ALL mice presented with different phenotypes of leukemic cells in different organs. We have demonstrated that the AML/ALL phenotype could present as AML in the bone marrow whereas the liver and spleen revelled a phenotype of ALL. We have shown earlier that deletion of PTEN may lead to expansion of $CD4^+$ lymphocytes in the bone marrow (Fig 3.9A) therefore speculate that despite the expansion of only myeloid cells in the blood, expansion of T- lymphocyte in other organs might have led to development of ALL. We did not see an expansion of B-lymphocyte and none of the mice came down with B-ALL. B-cell malignancies have not been associated with PTEN deletion or mutation. Moreover, a recent study by Shojaee and colleagues demonstrated that, loss of one or both alleles of PTEN caused swift cell death of pre-B ALL cells. PTEN deletion was also adequate to rescue transplant recipient mice from developing B-ALL (Shojaee et al., 2016). Studies using conditional PTEN KO mouse crossed with a Vav-Cre model to generate PTEN null mice demonstrated that these mice develop fatal disease with a median survival of 82 days (Tang et al., 2013). They further reported that these mice displayed a hunched posture, respiratory distress, ruffled fur and weight loss. When the diseased mice were dissected, a large thymic mass accompanied by hepatosplenomegaly was observed and histological examination of the thymus revealed an infiltration of the

thymus by monomorphic lymphoblastic cells. The presence of leukaemic cells in organs such as spleen and liver has also been reported. Similarly, our experimental mice that succumbed to leukaemia when PTEN alone was deleted in the bone marrow had a latency of around 80 days and they also showed comparable symptoms i.e. hunched back and respiratory distress. Moreover, FACS analysis of these mice also revealed the infiltration of leukaemic cells in the spleen and liver (Fig 4.7B, and Fig 4.8B).

The most important finding in the aforementioned studies is that PTEN deletion induced leukaemia is dependent on the chronic activation of PI3K/Akt signalling pathway. PDK1 controls the activation of substrates such as Akt1 and mTOR1 which have been shown to be key regulators in cell metabolism, proliferation, migration and survival (Li et al., 2010). Magee and colleagues compared the activation of Akt1 in HSCs/MPPs ($CD48^{-}$LSK) from E14.5 foetal, 8 weeks old adult wild type bone marrow and 8 weeks old bone marrow after PTEN deletion from Mx1 Cre; $PTEN^{fl/fl}$ mice. They showed that Akt was highly activated in PTEN deleted bone marrow compared to the normal bone marrow (Magee et al., 2012). These results suggest a different requirement for Akt activation during normal haematopoiesis and leukaemia development. Akt is the main activator of the mTOR complex 1 and it has been demonstrated that inhibiting the mTOR complex1 with rapamycin (Sarbassov et al., 2006, Guertin and Sabatini, 2007) rescued exhaustion of HSC and prevented leukaemogenesis upon loss of PTEN (Yilmaz et al., 2006b). Given their central roles within the PI3 kinase pathway, it is tempting to speculate that blocking the activation of Akt by inhibiting PDK1 could eliminate the development of LSCs and restore normal cell proliferation.

Interestingly when we conditionally deleted PDK1 alone, the PDK1 null cells failed to proliferate and died out in our transplant model suggesting that PDK1 has a critical role in cell survival and proliferation. Furthermore, we showed that simultaneous deletion of PTEN and PDK1 in the bone marrow impairs engraftment of PTEN null cells as demonstrated by a steady rate of proliferation (Fig 4.4A) suggesting a critical requirement for PDK1 in loss of PTEN induced proliferation. However, deletion of PDK1 was not sufficient to completely suppress PTEN induced leukaemia. It delayed the onset of disease with some of the transplanted mice remained disease free beyond 400 days. Biochemical testing of bone marrow cells from mice that succumbed to leukaemia when PTEN and PDK1 were deleted showed comparable phosphorylation of AKT Thr308, AKT Ser473 as well as SGK1 Ser206 to transplant mice that came down when PTEN alone was deleted. In the contrast, we have also shown that before the development of leukaemia deletion of PDK1 in PTEN deficient cells completely inhibited the phosphorylation of AKT Thr308 suggesting that PDK1 may be bypassed during leukaemogenesis leading idiopathic activation of AKT Thr in the absence of PDK1. It is also worth noting that when both PTEN and PDK1 were deleted, we only observed AML but not ALL. PTEN deletion mediated leukaemogenesis may bypass the normal requirement of PDK1 for activation of downstream PI3K pathway enzymes as a mechanism of cancer cell survival and growth. Together our finding suggest that AKT Thr308 phosphorylation triggered at some point during development of leukaemia and it is not mediated by PDK1. AKT functions may not be required during normal haematopoiesis but may be a requirement for developing leukaemia stem cells.

However, PDK1 cannot be ruled out as a key mediator of leukaemogenesis as its deletion completely cleared transplant mice from ALL and delayed latency of AML

induced by PTEN deletion. In another study using the Lck cre system which selective induces the deletion of PTEN alone or simultaneously deletion of PTEN and PDK1 only in T-cell progenitors. In these mice, the $p56^{lck}$ promoter mediates cre activation in T cell precursors at double negative (DN) 3 and 4 stage of lymphoid development before expression of the MHC receptor CD4 and CD8 (Finlay et al., 2009). It was reported that by deleting PTEN on its own the mice died of lymphoma in about 13 weeks (Finlay et al., 2009). Interestingly, when PTEN and PDK1 were deleted simultaneously in the lymphocytes, majority of the mice survived with no clinical symptoms of lymphoma (Finlay et al., 2009). Only a few mice with combined deletion of PTEN and PDK1 developed lymphoma and it was argued that the mice might have escaped the deletion of PDK1. Our experimental setup used a conditionally inducible Rosa-26cre-ERT system to induce deletion of PTEN and PDK1 in all of the haematopoietic cells after transplant. In contrast to Finlay and colleagues data, we showed that simultaneous deletion of PTEN and PDK1 did not induce T-ALL in recipient mice. However, the majority of lymphoid cells that caused the disease in Finlay's study originated from the earlier progenitors double positive population DP ($CD45^{+}CD25^{+}$). $CD4^{+}$ and $CD8^{+}$ lymphocytes were greatly reduced in $PTEN^{fl/fl}PDK1^{flfl}$Lck cre mice. Our data however showed that PTEN deletion in the bulk of the bone marrow resulted in T-ALL that was $CD4^{+}$ and $CD8^{+}$. This difference could be due to the activation of the Cre in different stages of haematopoietic development.

Surprisingly, mutations in PDK1 such as PDK1 MGK465 and PDK1 L155E disrupting the PH and PIF domain, respectively had little impact on loss of PTEN mediated cell proliferation. The PDK1 MGK465 mutation which does not allow the activation of Akt through PDK1 seemed to slightly delay the on-set of AML induced

by PTEN deletion, suggesting that Akt might play a role in leukaemogenesis. In contrast, the PDK1 L155 mutation which impairs the phosphorylation of S6K, SGK1 and RSK does not have any significant impact on the onset of AML induced by PTEN deletion but showed a slight delay of ALL onset suggesting that other kinases such as S6K, SGK1 and RSK have little or no role in loss of PTEN induced AML while contributing to ALL. These results suggest that AML and ALL might have different requirement for PDK1 signalling. In our mouse model, manipulation of PDK1 by introducing a mutation had no effect on activation of AKT Thr308 or SGK1 similar to what we observe with total ablation of PDK1 further supporting the speculation that PTEN deletion by passes the normal requirement for PDK1 to activate downstream effectors.

Chalhoub and colleagues demonstrated that PTEN deficiency caused macrocephaly in mice whereas PDK1 deficiency resulted in microcephaly showing an antagonistic effect. Interestingly combined deletion of PTEN and PDK1 fully rescued the PTEN deficient macrocephaly. Similarly, PDK1 deficiency rescued cell-autonomous hypertrophy of PTEN deficient neuronal nuclei and somata. However, the PDK1 deficiency did not rescue cerebellar neuron migration in PTEN deficient brains. Taken together these results provide evidence that loss of PDK1 reverts many loss of PTEN phenotypes in normal and malignant development, suggesting that PDK1 might be a starting point in targeting PTEN deficient cancer. However targeting PDK1 on its own may not fully restore the spectrum of pathology induced by loss of PTEN as PDK1 could not prevent abnormal migration of cerebral neurons nor could it reduce the invasiveness of malignant T-cells (Iwanami et al., 2009, Chalhoub and Baker, 2009). More importantly, Cantrell and colleagues demonstrated that PTEN null lymphocytes can increase in size, proliferate and differentiate without PDK1. This

shows that targeting PDK1 alone may not be enough to rescue leukaemic cells which may by-pass the normal PDK1 requirements for cell growth and proliferation. One important question is whether Akt is still required for maintenance of leukaemic stem cells as previously suggested. Several studies have shown that the expression of phosphorylated Akt is increased upon PTEN deletion (Lim et al., 2014, Cai et al., 2014, Carnero and Paramio, 2014, Kim et al., 2014).

CHAPTER 5 PI3K/PDK1/AKT/PKC inhibitors

5.1 Brief Introduction

Targeting the PI3K signalling network has been shown to be of therapeutic value in the treatment of cancer (Fransecky et al., 2015). We have demonstrated earlier that loss of PTEN mediated leukaemogenesis can be delayed by simultaneously silencing PDK1. Under normal condition, PDK1 is responsible for activation of downstream kinases such as AKT, SGK1, RSK and PCK. Noticeably, PDK1 is the only kinase known to phosphorylate AKT Thr308. While deletion of PDK1 in $PTEN^{-/-}$ cells did initially inhibit phosphorylation of downstream kinases including pAKT308Thr, AKT and few other substrates were subsequently phosphorylated when compound PTEN /PDK1 KO became leukaemic. This not only suggests that AKT Thr308 plays an important role in leukaemogenesis mediated by PTEN deficiency, but also demonstrates that leukaemic cells can bypass the normal requirement for PDK1 to phosphorylate and activate AKT Thr308. Crosstalk between PI3K and MAPK/ERK has been previously reported (Zhou et al., 2015, Mendoza et al., 2011). We therefore speculate that AKT Thr308 may be activated in response to PDK1 deficiency as a result of novel crosstalk with other pathways such as MAPK and ERK in leukaemogenesis. The kinase(s) responsible for the phosphorylation of AKT Thr308 in the absence of PDK1 has not been documented and remains completely unknown.

To this end, we sought to set up an in vitro assay to pursue a candidate approach and also potentially a high throughput kinase inhibitor screen to identify the

kinase(s) that might be responsible for the observed phosphorylation of AKT Thr308. As PTEN and PDK1 deficient leukaemic cells do not grow very well in in vitro cultures, I decided to use mouse embryonic fibroblasts (MEFs) instead and investigated the effects of different inhibitors targeting different components of the PI3K pathway in PTEN deficient MEFs and compound PTEN and PDK1 deficient MEFs. We generated PTEN MEFs by setting up time matings with $PTEN^{fl/fl}$Rosa-26cre-ERT male and $PTEN^{fl/fl}$ female. To produce compound PTEN and PDK1 MEFs, we set up time matings with $PTEN^{fl/fl}PDK1^{fl/fl}$ Rosa-26cre-ERT male and $PTEN^{fl/fl}PDK1^{fl/fl}$ female. MEFs were harvested at E13.5 days and cultured in DMEM medium supplemented with 10% foetal bovine serum. We allowed at least 3 passages before treating with 10 µM of tamoxifen for 2 to 4 days to induce the deletion of PTEN and PDK1 (Fig 5.1).

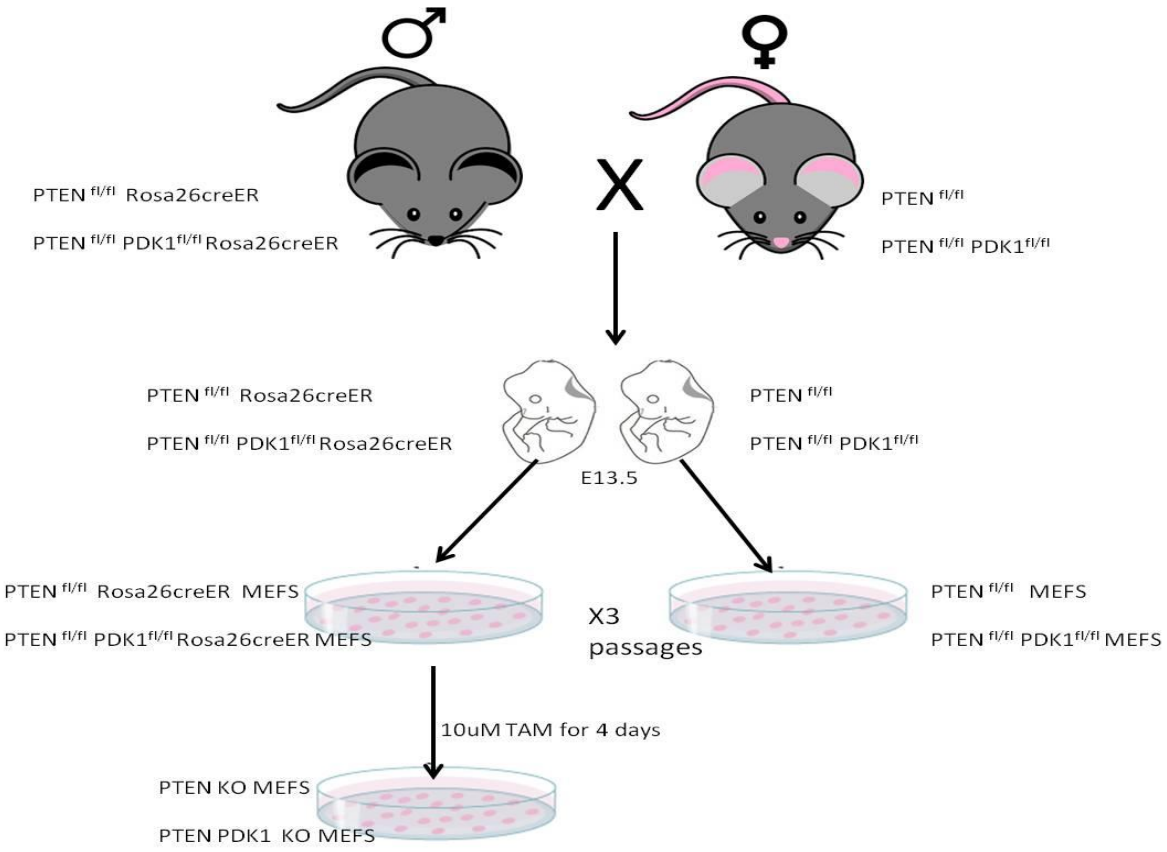


Figure 5.1 Generation of PTEN and PDK1 null MEFs.

Timed mating set up with $PTEN^{fl/fl}$Rosa-26cre-ERT male and $PTEN^{fl/fl}$Rosa-26cre-ERT to generate PTEN KO MEFs. Timed mating set up with $PTEN^{fl/fl}PDK1^{fl/fl}$Rosa-26cre-ERT male and $PTEN^{fl/fl}PDK1^{fl/fl}$Rosa-26cre-ERT female to generate compound PTEN and PDK1 KO MEFs.

5.1.1 PDK1 inhibitor (GSK2234470) has no effect on AKT Thr308 phosphorylation induced by PTEN deletion

We have earlier demonstrated that AKT is phosphorylated at Thr308 in the absence of PDK1 in PTEN deficient leuakemic cells (Fig 4.12). To determine if we will obtain similar responses in wild type and PTEN KO MEFs, we used a PDK1 inhibitor (GSK2234470) mimicking the effects observed in PDK1 KO. Wild type and PTEN deleted MEFs were treated for 1 hour *in vitro* with increasing doses of the PDK1 inhibitor, and cell lysates were subsequently prepared and subjected to western blot. As a result, at concentrations as little as 1.0 μM, the inhibitor completely inhibited the activity of PDK1 in the wild type MEFs as shown by loss of phosphorylation of AKT Thr308 (Fig 5.2A). Interestingly, PDK1 inhibitor did not impair the phosphorylation of AKT Thr308 even at the highest concentration tested (10 μM) in PTEN deleted MEFs (Fig 5.2B), strongly suggesting that deletion of PTEN bypasses the normal requirement for PDK1 to activate certain downstream effectors such as AKT Thr308 in the PI3 kinase pathway. As expected, the inhibitor did not have any effect on the phosphorylation of AKT Ser473, which is mediated by mTOR2 and therefore cannot be affected by inhibition of PDK1. Phosphorylation of SGK1 Thr256 was slightly reduced in wild type MEFs with doses from 5 μM to 10 μM, but there was no effect on PTEN

null MEFs, indicating that SGK1 may also be phosphorylated in the absence of PDK1 in the PTEN$^{-/-}$ background.

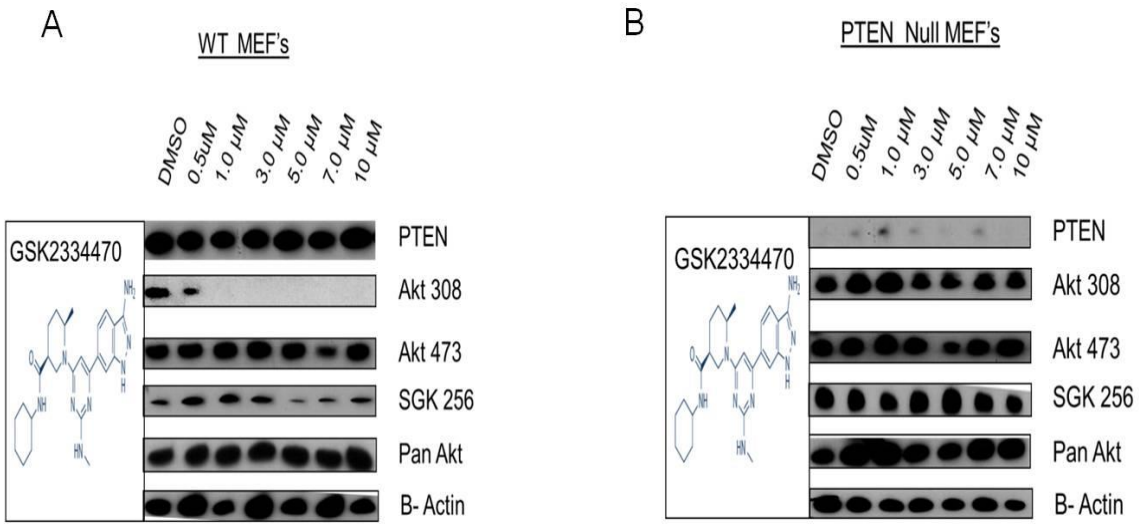


Figure 5.2 Immuno-blotting of PDK1 down-stream effectors after treatment with GSK2334470 PDK1 inhibitor.

The indicated MEFs treated for 1 hour with GSK2334470 PDK1 inhibitor were lysed and subjected western blot with antibodies specific for the indicated down-stream effectors of PDK1. (A) Wild type MEFs treated with inhibitor. (B) PTEN deleted MEFs treated with inhibitor. (lane 1) DMSO; (lane 2) 0.5 µM of inhibitor; (lane 3) 1.0 µM of inhibitor; (lane 4) 3.0 µM of inhibitor; (lane 5) 5.0 µM of inhibitor; (lane 6) 7.0 µM of inhibitor; (lane 7) 10.0 µM of inhibitor.

5.1.2 AKT VIII inhibitor has no effect on phosphorylation of AKT Thr308 in the absence of PDK1 in PTEN deleted MEFs

Phosphorylation of AKT at the activation loop (Thr308) is mediated by PDK1 through the interaction of the PH domain under normal conditions. To test the possibility that activation of AKT Thr308 is mediated by interaction with residual PDK1 protein that could have remained after deletion or a conformational change in the folding of the AKT enzyme that may lead to auto phosphorylation, we used AKT VIII inhibitor, which is a reversible quinoxaline compound that potently and selectively inhibits Akt1/Akt2 activity. The inhibition activity of AKT VIII prevents the conformational chance of AKT and is PH domain depent therefore has no effect against PH domain-

lacking Akts, or other closely related AGC family kinases, PKC, and SGK, even at very high concentration. Compound PTEN and PDK1 deleted MEFs as well as wild type MEFs (control) were treated for 1 hour *in vitro* with increasing doses of the AKT VIII inhibitor and cell lysates were subsequently prepared and subjected to western blot. We demonstrate that as little as 0.5 μM of AKT VIII inhibitor was able to inhibit the activity of AKT Thr308 in wild type MEFs whereas even at the highest concentration of this inhibitor tested (10 μM) did not impair the phosphorylation of AKT Thr308 in PTEN and PDK1 deleted MEFs (Fig5.3). These results further strengthen the idea that a kinase other than PDK1 may be responsible for the phosphorylation of AKT Thr308. As expected AKT VIII inhibitor did not show any effect on AKT Ser473, which is mediated by mTORC.

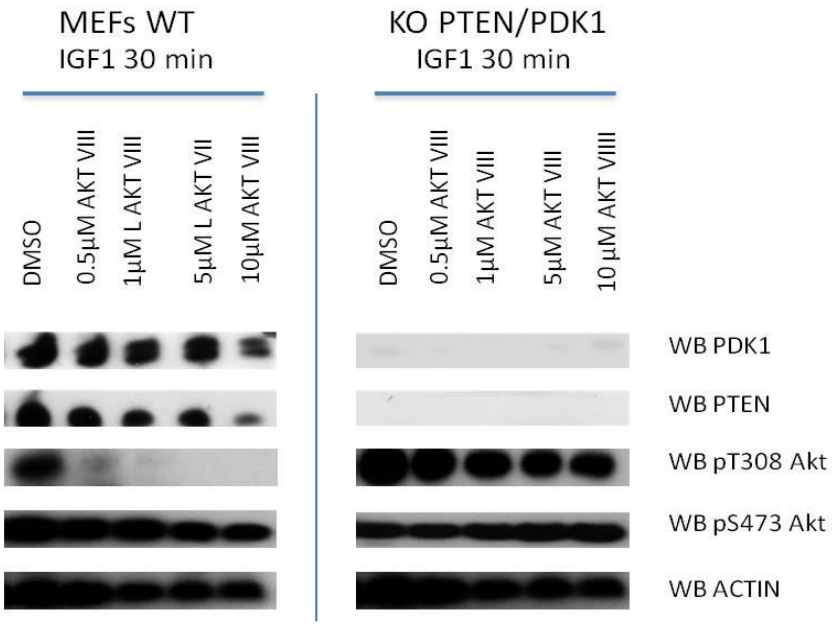


Figure 5.3 Immuno-blotting of PDK1 down-stream effectors after treatment with AKT VIII inhibitor.

Compound PTEN and PDK1 deleted MEFs as well as wild type MEFs (control) were treated for 1 hour *in vitro* with increasing doses of the AKT VIII inhibitor and cell lysates were subsequently prepared and subjected to western blot with antibodies specific for the indicated down-stream effectors of PDK1. (A) Wild type MEFs treated with inhibitor. (B) PTEN and PDK1 deleted MEFs treated with inhibitor. (lane 1) DMSO; (lane 2) 0.5 μM of inhibitor; (lane 3) 1.0 μM of inhibitor; (lane 4) 5.0 μM of inhibitor; (lane 5) 10.0 μM of inhibitor.

5.1.3 PKC inhibitor (817) has no effect on phosphorylation of AKT Thr308 in the absence of PDK1 in PTEN deleted MEFs

To further investigate the probable cause of phosphorylation of AKT Thr308 in the absence of PDK1 in PTEN null MEFs, we used a PKC inhibitor to test if the phosphorylation of AKT Thr308 could be a result of feedback loop mediated by PKC. Compound PTEN and PDK1 deleted MEFs as well as wild type MEFs (control) cultured in a six well plate were treated for 1 hour with increasing doses of the PKC inhibitor, and cell lysates were subsequently prepared and subjected to western blot. Interestingly, PKC inhibitor was able to impair the phosphorylation of AKT Thr308 in wild type MEFs suggesting that it has anti PDK1 or AKT activity although directly targeting PKC. On the other hand, PKC inhibitor was not able to inhibit the phosphorylation of AKT Thr308 in PTEN and PDK1 deleted MEFs (Fig 5.4). These results suggest that PKC is unlikely the kinase that mediates the phosphorylation of AKT Thr308 in the absence of PDK1.

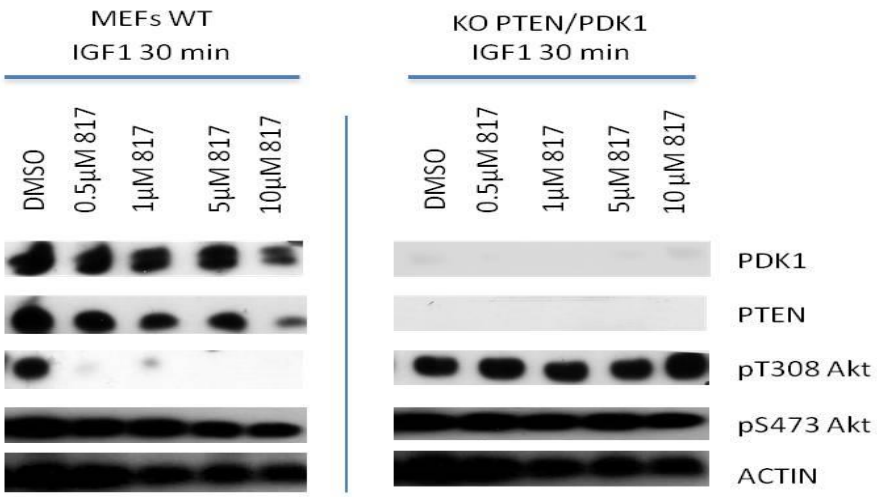


Figure 5.4 Immuno-blotting of PDK1 down-stream effectors after treatment with PKC inhibitor (817).

Compound PTEN and PDK1 deleted MEFs as well as wild type MEFs (control) were treated for 1 hour *in vitro* with increasing doses of the PKC-i inhibitor and cell lysates were subsequently prepared and subjected to western blot with antibodies specific for the indicated down-stream effectors of PDK1. (A) Wild type MEFs treated with inhibitor. (B) PTEN and PDK1 deleted MEFs treated with inhibitor. (lane 1) DMSO; (lane 2) 0.5 µM of inhibitor; (lane 3) 1.0 µM of inhibitor; (lane 4) 5.0 µM of inhibitor; (lane 5) 10.0 µM of inhibitor.

5.1.4 PI3K inhibitor LY294002 completely inhibited the phosphorylation of AKT Thr308 in compound PTEN and PDK1 deleted MEFs

We next tested the effect of PI3K inhibitor LY294002 on compound PTEN and PDK1 deleted MEFs. LY294002 is a flavonoid derivative reversible inhibitor and has been shown to effectively target a wide range of kinases including all isoforms of PI3K. We cultured compound PTEN and PDK1 deleted MEFs in a six well plate, and treated with increasing doses of the LY294002 for 1 hr once they reached 90% confluence. Wild type MEFs were subjected to similar treatments. We observed a decreased phosphorylation of AKT Thr308 in wild type MEFs treated with 0.5 µM of LY294002 inhibitor, and a complete inhibition was achieved with 1 µM. Interestingly, we also observed a reduction of phosphorylation of AKT Thr308 in compound PTEN and

PDK1 null MEFs treated with 0.5 µM of LY294002 inhibitor and a complete inhibition of AKT Thr308 phosphorylation with 1 µM of LY294002 comparable to what we observed in wild type MEFs (Fig 5.5). These results suggest that targeting of PDK1 together with PI3K or closely related kinases might be required for complete inhibition of AKT Thr308 in PTEN$^{-/-}$ cells. Noticeably, LY294002 also slightly inhibited the phosphorylation of AKT Ser473 at doses from 5 µM to 10 µM in wildtype MEFs and compound PTEN and PDK1 deleted MEFs, suggesting that it has effect on mTOR2 activity, which is known to directly activate AKT Ser473.

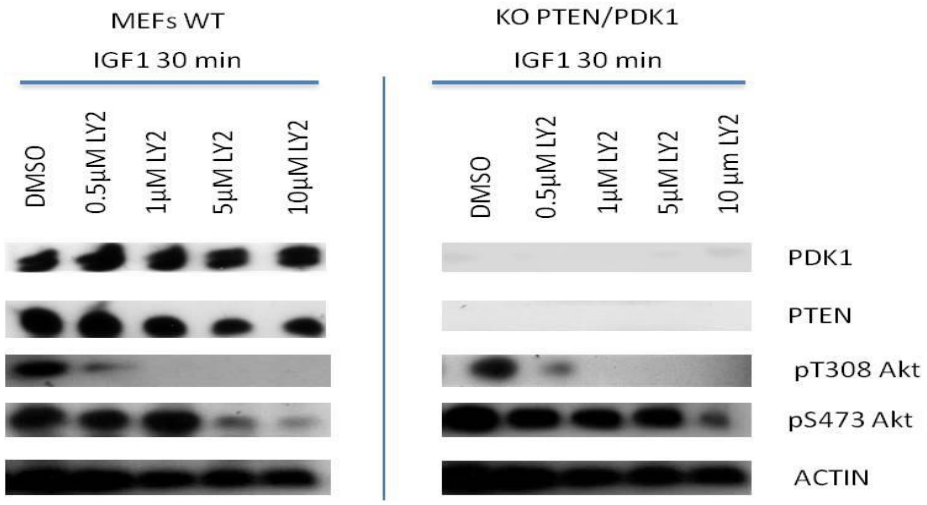


Figure 5.5 Immuno-blotting of PDK1 down-stream effectors after treatment with Pi3K inhibitor LY294002.

Compound PTEN and PDK1 deleted MEFs as well as wild type MEFs (control) were treated for 1 hour in vitro with increasing doses of the LY294002 inhibitor and cell lysates were subsequently prepared and subjected to western blot with antibodies specific for the indicated down-stream effectors of PDK1. (A) Wild type MEFs treated with inhibitor. (B) PTEN and PDK1 deleted MEFs treated with inhibitor. (lane 1) DMSO; (lane 2) 0.5 µM of inhibitor; (lane 3) 1.0 µM of inhibitor; (lane 4) 5.0 µM of inhibitor; (lane 5) 10.0 µM of inhibitor.

5.1.5 PI3K inhibitor LY294002 could not completely inhibit the phosphorylation of AKT Thr308 in PTEN deleted MEFs

We have shown earlier that targeting PI3K with LY294002 resulted in a complete inhibition of AKT Thr308 phosphorylation in PTEN and PDK1 deleted MEFs, suggesting that PI3K or closely related kinase sensitive to LY294002 might be responsible for the phosphorylation of AKT Thr308 in the absence of PDK1. We next would like to test if LY294002 could also successfully inhibit phosphorylation of AKT Thr308 when PTEN has been deleted but PDK1 is fully functional in MEFs. We set up and dose time response experiment by treating PTEN deleted MEF' with 50 μM of LY294002 for 10, 20, 30, 40 and 60 minutes. We demonstrate that LY294002 partially inhibited the phosphorylation of AKT Thr308 when exposed to treatment for 10 to 20 minutes (Fig 5.6). Interestingly phosphorylation of AKT Thr308 was regained at 30 minutes. The reappearance of phosphorylation could be a result of feedback that counteracts the effect of PI3K inhibition. These results suggest that targeting PI3K alone is not sufficient to counteract the constitutive activation of AKT caused by PTEN deletion, unless PDK1 was also inactivated.

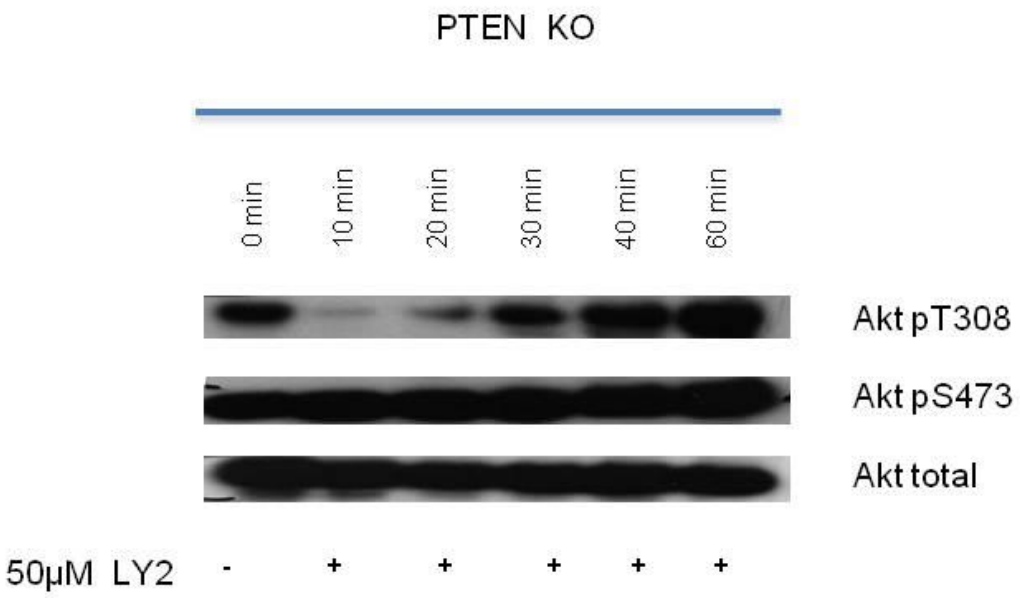


Figure 5.6 Dose time responses of AKT Thr308 and AKT Ser473 after treatment with LY2942002 inhibitor.

PTEN deleted MEFs treated with 50 μM of LY294002 for 10, 20, 30, 40 and 60 minutes.

5.1.7 Activation of AKT Thr308 may be dependent on both PI3K and PDK1 in PTEN deleted MEFs

To test if the phosphorylation of AKT Thr308 is dependent on PI3K or PDK1 when PTEN is deleted, we set up a dose vs time response using PDK1 inhibitor (GSK2234470) after pre-treatment with PI3K inhibitor (LY294002). Firstly, we treated PTEN deleted MEFs with 50 μM of LY294002 for 30 minutes, then we aspirated the medium and replaced with fresh medium containing 1 μM of GSK2234470 for 10, 20, 30, 40 and 60 minutes. Interestingly, treatment with the PDK1 inhibitor after pre-treatment with LY294002 inhibited the phosphorylation of AKT Thr308 in the first 10 minutes. Phosphorylation was partially regained from 20 to 30 minutes and fully regained post 40 minutes treatment (Fig 5.7). This suggests

that the combined effect of two inhibitors could potentially inhibit the phosphorylation of AKT Thr308 in PTEN deleted MEFs. The gradual regain of AKT Thr308 phosphorylation could be due to recovery from the earlier exposure to LY294002, which is reversible upon removal of the inhibitor. Full phosphorylation of AKT Thr308 in the presence of PDK1 inhibitor also indicates that inactivation PDK1 is not sufficient to inhibit AKT Thr308 phosphorylation when PTEN is deleted in MEFs. We did not observe any effect on the phosphorylation AKT Ser473 in this experimental set up.

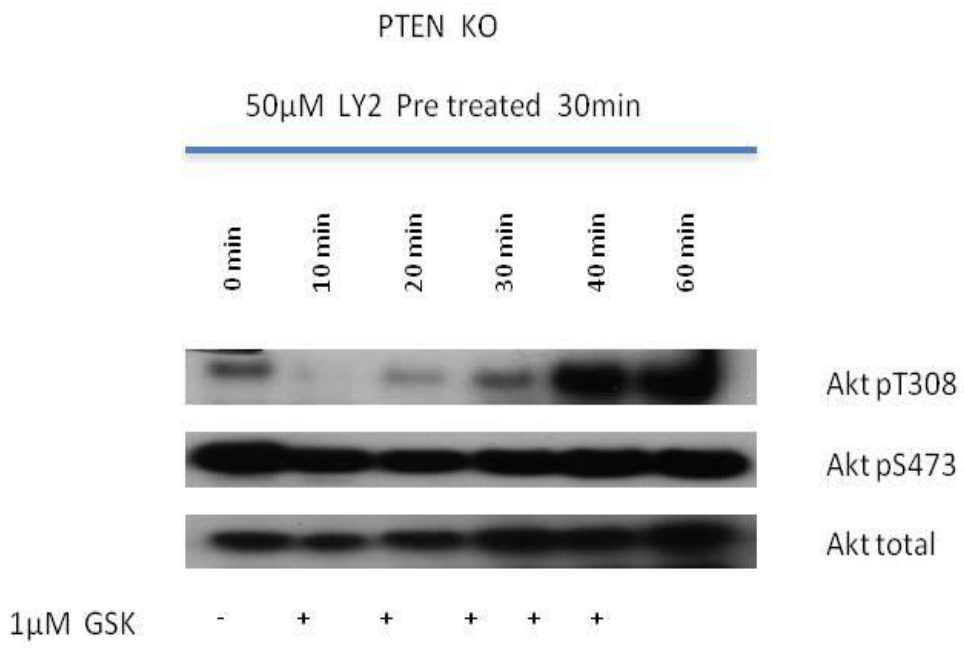


Figure 5.7 Dose time responses of AKT Thr308 and AKT Ser473 pre-treated LY2942002 inhibitor and treated with GSK2234470.

PTEN deleted MEFs treated with 50 µM of LY294002 for 30 minutes and aspirated to replace with medium containing 1 µM GSK2234470. Analysis was carried out at 0, 10, 20, 30, 40 and 60 minutes.

5.1.8 Dual treatment of PTEN deleted MEFs with LY294002 and GSK2234470 inhibitors resulted in short term inhibition of AKT Thr308

We have shown earlier that when we treated compound PTEN and PDK1 deleted MEFs with PI3K inhibitor (LY294002), we observed a complete inhibition of AKT Thr308 with doses below 10 µM. We further showed that pre-treating PTEN deleted MEFs with LY294002 and treating with PDK1 inhibitor (GSK2234470) successfully inhibited the phosphorylation of AKT Thr308 in the first 10 minutes. To further confirm the effect of dual inhibition of PI3K and PDK1 in PTEN deleted MEFs, we set up a dose time response by treating PTEN deleted MEFs with 50 µM of LY294002 and 1 µM of GSK2234470. In contrast to the pre-treatment strategy, dual treatment with LY294002 and GSK2234470 completely inhibited the phosphorylation of AKT Thr308 in the observed time frame (10 to 60 minutes). Although low levels of AKT Thr308 phosphorylation might be regained past 60 minutes, these results suggest that combined treatment of LY294002 and GSK2234470 completely inhibits pAKT308Thr for at least 60 minutes.

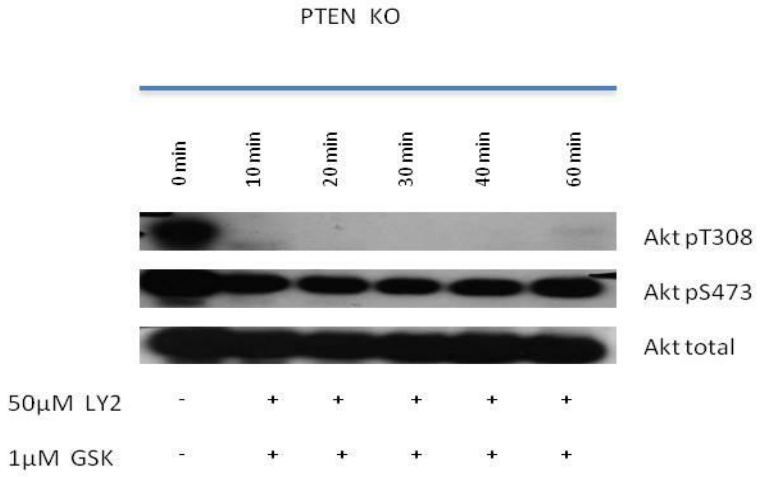


Figure 5.8 Dose time responses of AKT Thr308 and AKT Ser473 after dual treatment with LY2942002 GSK2234470 inhibitor.

PTEN deleted MEFs treated with 50 µM of LY294002 and 1 µM GSK2234470. Analysis was carried out at 0, 10, 20, 30, 40 and 60 minutes.

5.1.9 Pan-PI3K inhibitor suppresses the activation of AKT Thr308 in PTEN deleted MEFs

We have demonstrated earlier that the PI3K inhibitor LY294002 showed some effective short term inhibition of AKT Thr308 in the first 10 minutes of treatment. Moreover dual treatment with LY294002 and GSK2234470 exhibited very effective inhibition of AKT Thr308. We next tested another PI3K inhibitor (NVP-BKM120) which is a pan-PI3K inhibitor capable of inhibiting all isoforms of class 1 PI3K (PI3Kα, PI3K, PI3Kβ, PI3Kδ, and PI3Kγ), as well as the individual isoform inhibitors. We treated PTEN deleted MEFs with 1 μM pan-PI3K inhibitor and 1 μM of each of the individual isoforms inhibitors (PI3Kα inhibitor, PI3Kβ inhibitor, PI3Kδ inhibitor, and PI3Kγ inhibitor) for 1 hour prior to preparing cell lysates. Interestingly, we observed that the pan-PI3K inhibitor greatly suppressed the phosphorylation of both AKT Thr308 and AKT Ser473 (Fig 5.9). Surprisingly, the individual isoform inhibitors had no effect on the phosphorylation of either AKT Thr308 or AKT Ser473. The down regulation of AKT Ser473 suggests that the pan-PI3K inhibitor might also have effect on mTOR2 similarly to LY294002 inhibitor. Together, these results suggest that a negative feedback loop involving multiple PI3K isoforms, mTOR2 or other related kinase might be responsible for the phosphorylation of AKT Thr308 in the absence of PDK1 in PTEN deleted cells.

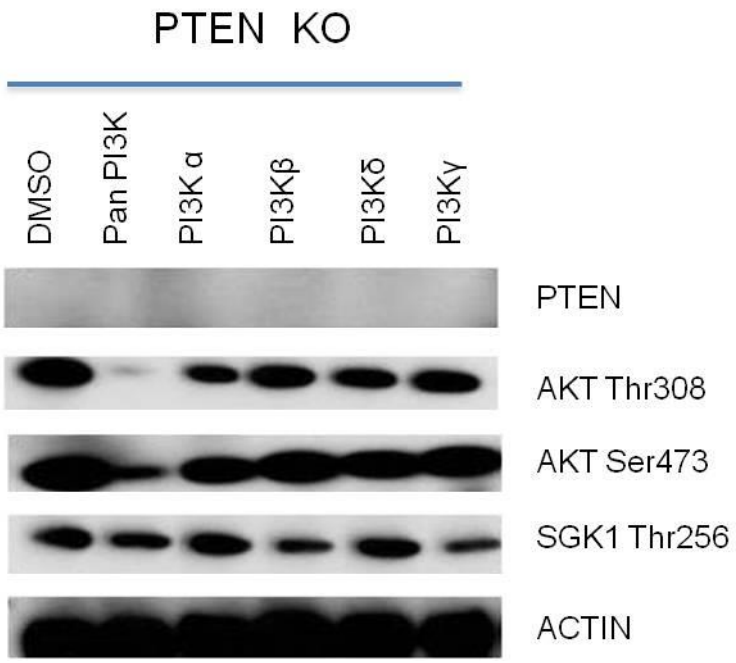


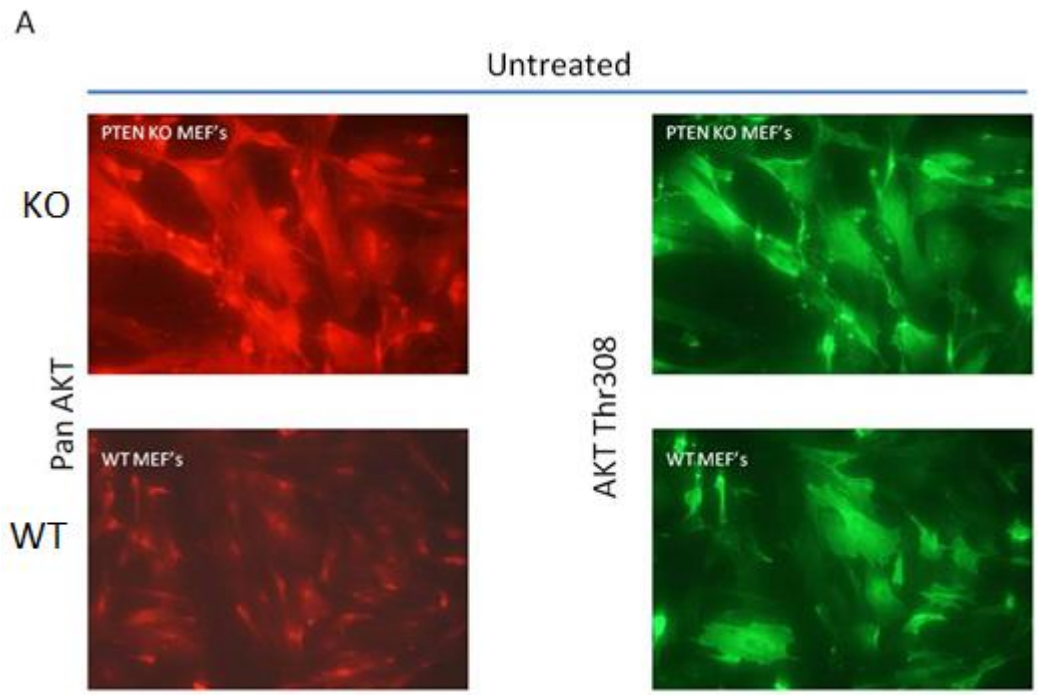
Figure 5.9 Immuno-blotting of PDK1 down-stream effectors after treatment with pan-PI3K inhibitors and isoform inhibitors.

PTEN deleted MEFs with 1 μM pan-PI3K inhibitor and 1 μM of each of the individual isoforms inhibitors (PI3Kα inhibitor, PI3Kβ inhibitor, PI3Kδ inhibitor, and PI3Kγ inhibitor) for 1 hour prior to preparing cell lysates. Western blot analysis for AKT Thr308/Ser473 and SGK1 Thr256. (lane 1) DMSO; (lane 2) 1 μM of pan-PI3K inhibitor; (lane 3) 1 μM of PI3Kα inhibitor; (lane 4) PI3Kβ inhibitor; (lane 5) 1 μM of PI3Kδ inhibitor; (lane 6) 1 μM of PI3Kγ inhibitor.

5.2 Analysis of AKT Thr308 activity using immunofluorescence in PTEN MEFs and wild type MEFs treated with PDK1 inhibitor

To identify signalling mechanism and the kinases underlying the phosphorylation of AKT Thr308 in the absence of PDK1 in PTEN deleted cells, we reasoned that a high throughput kinase inhibitor screen may provide an unbiased and global search for such kinases/mechanisms. Due to the complexity of the kinase inhibitor screen, western blot cannot be used to identify the potential hits and therefore immunofluorescence (IF) staining was to be developed for the screen. After demonstrating by western blot that the PDK1 inhibitor (GSK2234470) can effectively inhibit AKT Thr308 in wild type MEFs, I tested this PDK1 inhibitor also by IF. To this end, PTEN deleted and

wild type MEFs were seeded at $3x10^5$ cells per well in a six well plate with gelatine coated round cover slips. Treatment with 1 µM GSK2234470 (PDK1 inhibitor) was administered in half of the wells and the other left untreaded for 1 hour. We analysed the cells growing over the cover slip for expression of AKT Thr308. We demonstrated that AKT Thr308 could be detected in both PTEN deleted MEFs and wild type MEFs (5.10A). As expected treatment with 1 µM GSK2234470 had no effect on AKT Thr308 phosphorylation in PTEN deleted MEFs whereas phosphorylation of AKT Thr308 was completely inhibited in wild type MEFs (Fig 5.10B). These results are consistent with our earlier experiments using western blot, indicating the validity of using IF for assessment of AKT Thr308 status in a high throughput setting.



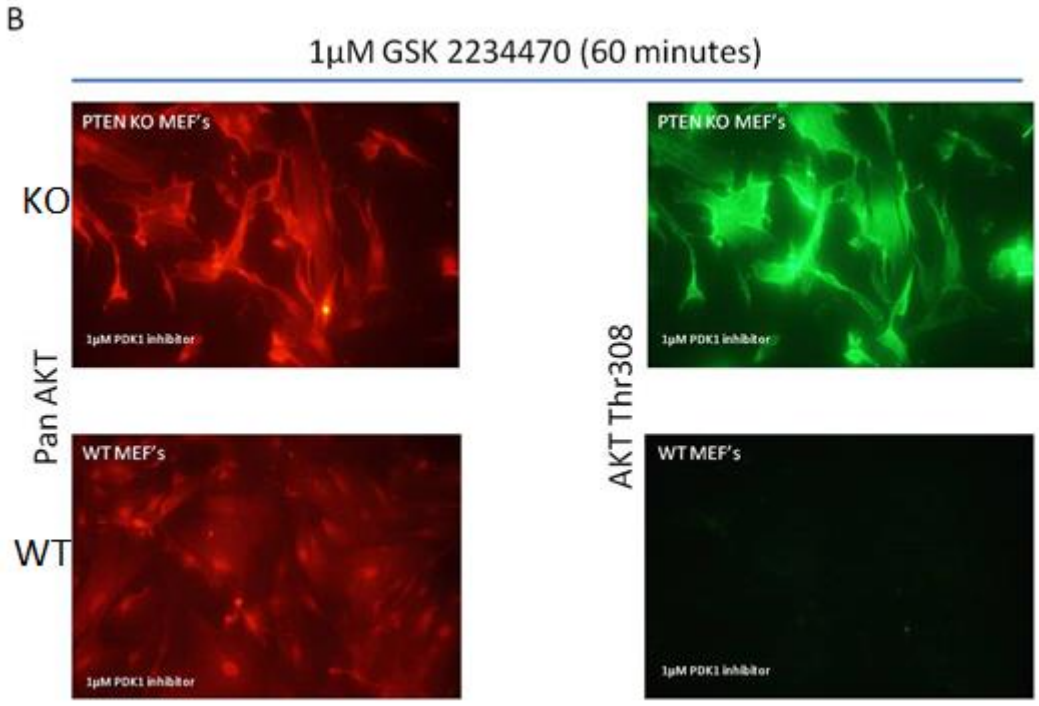


Figure 5.10 Immunofluorescence for AKT Thr308 and total AKT in PTEN deleted MEFs and WT MEFs.

PTEN deleted and wild type MEFs were seeded at $3x10^5$ cells per well in a six well plate with gelatine coated round cover slips. Treatment with 1 µM GSK2234470 (PDK1 inhibitor) was administered in half of the wells and the other left unthreaded for 1 hour. (A) We demonstrate that AKT Thr308 could be detected in both PTEN deleted MEFs and wild type MEFs. (B) Treatment with 1 µM GSK2234470 had no effect on AKT Thr308 phosphorylation in PTEN deleted MEFs whereas phosphorylation of AKT Thr308 was completely inhibited in wild type MEFs.

Discussion

Our genetic KO data showed that deletion of PTEN resulted in constitutively activated AKT Thr308 that could not be inhibited by deletion of PDK1, suggesting that the normal requirement of PDK1 for activation of AKT may be bypassed in the absence of PTEN. To support this data, we treated wild type MEFs with PDK1 inhibitor GSK2334470, and showed that we can completely inhibit phosphorylation of Akt at T308 but not at S473. Interestingly when we treated PTEN null MEFs with PDK1 inhibitor GSK2334470, we could not inhibit the phosphorylation of Akt at T308 even with the maximum dose of 10 μM of the inhibitor, suggesting that PTEN deletion might trigger other pathways that might lead to phosphorylation of Akt independent of PDK1. We tested AKT VIII inhibitor which selectively target AKT1 and AKT2 with a mechanism of action dependent on the presence of the PH domain of the full-length protein (Calleja et al., 2009). AKT inhibitor VIII targets the AKT inactive conformer preventing conformational change of AKT resulting in loss of AKT Thr308 phosphorylation (Calleja et al., 2009). We demonstrate that the AKT VIII did not have any effect on AKT Thr308 in PTEN and PDK1 deleted cells, suggesting that constitutive activation of AKT Thr308 in $PTEN^{-/-}$ cells is not attributed to conformational change and therefore rules out the possibility of auto phosphorylation or phosphorylation of AKT by residual PDK1. AKT VIII prevents the conformational change of AKT therefore preventing phosphorylatyion of AKT via the PH domain as well as auto phosphorylation. The PKC inhibitor (817) did not have any effect on phosphorylation of AKT Thr308 ruling out the possibility of a feedback effect that might evolve PKC for the phosphorylation of AKT Thr308 when both PTEN and PDK1 have been lost.

Finally, we tested the PI3K inhibitor (LY294002) and showed that it was able to inhibit AKT Thr308 in compound PTEN and PDK1 deleted MEFs. Interestingly, the LY294002 showed slight inhibition of AKT Ser473 in compound PTEN and PDK1 deleted MEFs treated with 10 μM of inhibitor, suggesting that the inhibitor may also have effect on mTOR2 which mediated the phosphorylation of AKT Ser473. However, LY294002 inhibitor failed to inhibit the phosphorylation of AKT Thr308 when PTEN alone was deleted in MEFs. We further went on to test another PI3K inhibitor and specific PI3K isoform inhibitors. We showed that the specific isoform inhibitors (PI3Kα, PI3K, PI3Kβ, PI3Kδ, and PI3Kγ) had no effect on AKT Thr308 phosphorylation. Intriguingly, the pan-PI3K inhibitor (NVP-BKM120) capable inhibiting all class 1 PI3K isoforms greatly inhibited AKT Thr308 as well as AKT Ser473 in PTEN deleted MEFs. LY294002 and pan-PI3K inhibitor have effect on wide range of other kinases, and therefore it is inconclusive to pick PI3K as the kinase responsible for the phosphorylation of AKT Thr308 in the absence of PDK1. Together these results suggest that targeting of multiple different kinases is required for the successful reversion of the impacts by PTEN deletion.

Previous studies have shown that the mTORC1 is an effector of the PI3 kinase pathway that promotes cell growth, proliferation and survival. It has also been shown that chronic stimulation of the mTORC1 leads to phosphorylation and down regulation of IRS1, which in turn impairs the activation of PI3 kinase as a negative feedback mechanism (Zhang et al., 2007, Sarbassov et al., 2005, Sabatini, 2006). Insight into signal transduction pathways over the past years have shown that no single pathway operates in isolation and that pathways are small parts of fully integrated networks within the cell (Manning and Cantley, 2007). Therefore it is conceivable a functional crosstalk of the PI3 kinase pathway with other pathways that might lead to

activation of Akt independent from PDK1 in PTEN deletion mediated cancer. The most common pathway known to have cross talk with the PI3 kinase pathway and also contribute to cell growth, proliferation and survival is the Ras/Erk/Mek pathway (Carracedo and Pandolfi, 2008). The Ras/Erk/ Mek pathway has been shown to activate RSK and S6K1 as well as inhibit the TSC1/2 leading to the activation of mTORC1 (Chappell et al., 2011). More research including high throughout screen has to be done to investigate these crosstalk mechanisms that might contribute to cancer cell survival and drug resistance.

CHAPTER 6

Conclusions

The PI3K pathway has been associated with many cellular functions such as cell metabolism, cell growth, survival, proliferation and drug resistance. Substantial data suggest that the PI3K pathway is often perturbed in a number of haematological malignancies as well as other forms of cancers (Barrett et al., 2012). Targeting this pathway may be of therapeutic value in the treatment of haematological malignancy and cancer in general. Many drugs targeting different enzymes of the PI3K pathway have been designed and some of them tested in clinical trial (Dancey, 2010). Although promising results have been gathered on the effectiveness of these drugs, remission of cancer and drug resistance is always almost inevitable. The mechanism of cancer and resistance to drug is still not fully understood and therefore further insights need to be gathered. The main goal of my PhD was to investigate the role of PTEN and PDK1 in leukaemogenesis and normal haematopoiesis using mouse models where I can simultaneously delete PTEN and PDK1 in haematopoietic cells. To demonstrate the significance of PTEN and PDK1 in haematopoiesis, we showed that deletion of PTEN depleted normal haematopoietic stem cells and early progenitors. Simultaneous deletion of PTEN and PDK1 in haematopoietic cells was able to rescue some of the phenotype cause by PTEN deficiency strongly suggesting inhibition of PDK1 could be of therapeutic value in loss of PTEN mediated diseases.

Loss of PTEN has been shown to cause constitutive activation of the PI3K pathway and in the case of haematopoietic cells leads to the development of cancer. We demonstrate that loss of PTEN in the haematopoietic compartment results in the

development of AML or ALL and in some cases both AML and ALL consistent with previous published data. We further demonstrated that ablation of PDK1 in loss of function of PTEN in haematopoietic cells does not completely reverse the leukaemogenesis induced by PTEN deletion; however it was able to significantly delay the onset of AML. Moreover, deletion of PDK1 in PTEN deficient haematopoietic cells rescued the mice from developing ALL in both $PTEN^{fl/fl}PDK1^{fl/fl}$Rosa-26cre-ERT mice and $PTEN^{fl/fl}$ $PDK1^{fl/fl}$ Vav-cre mice.

We used distinctive PDK1 mutants to further elucidate the function of PDK1 in leukaemogenesis. We demonstrated that the PDK1 MGK465 mutation that disrupts the PH domain of PDK1 resulting in deficient activation of the AKT Thr308 slightly delayed the onset of AML in PTEN deleted haematopoietic cells and had no significant effect on the onset of ALL. On the other hand, the PDK1 L155 mutation that disrupts the PIF pocket resulting in deficient activation of S6K, RSK, SGK and PKC showed slight delay in the onset of ALL while it had no significant effect on the onset of AML. These results signify the importance of PDK1 as a target in the treatment of PTEN deletion induced leukaemogenesis. In addition, our data show that AML and ALL might have different requirements for downstream PDK1 targets and therefore it is important to understand the biology of the underlying pathway dysregulation in these conditions in order to decide on the treatment to use in a given disease. We hypothesize that the leukaemia that develop in the absence of PDK1 when there is loss of PTEN may have resulted from the bypassing the normal requirement of PDK1 to activate downstream targets. We use MEFs deficient for PTEN or deficient for both PTEN and PDK1 to investigate the underlying mechanism for the phosphorylation of AKT Thr308 in the deficiency of PDK1. We demonstrated that inhibitors such as LY294002 that target multiple kinases in combination with

inhibition of PDK1 are more efficient in inhibiting the phosphorylation of AKT Thr308. These results suggest targeting multiple kinase maybe the best approach to prevent drug resistance due to feedback or bypassing of the targeted kinase by other mechanisms. PIK3K pathway was previously shown to crosstalk with other pathways such as MAPK and ERK/Ras pathways which may contribute to the bypassing of PDK1 in PTEN deficiency. An important future step will be to carry out a high throughput kinase inhibitor screen to narrow down the kinases that might be responsible for the phosphorylation of AKT Thr308 in the absence of PDK1 when there is PTEN loss of function.

REFERENCES

ADOLFSSON, J., BORGE, O. J., BRYDER, D., THEILGAARD-MONCH, K., ASTRAND-GRUNDSTROM, I., SITNICKA, E., SASAKI, Y. & JACOBSEN, S. E. 2001. Upregulation of FLT3 expression within the bone marrow Lin(-)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. *Immunity,* 15**,** 659-69.

ALHARBI, R. A., PETTENGELL, R., PANDHA, H. S. & MORGAN, R. 2013. The role of HOX genes in normal hematopoiesis and acute leukemia. *Leukemia,* 27**,** 1000-8.

ANTONCHUK, J., SAUVAGEAU, G. & HUMPHRIES, R. K. 2002. HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. *Cell,* 109**,** 39-45.

BALENDRAN, A., HARE, G. R., KIELOCH, A., WILLIAMS, M. R. & ALESSI, D. R. 2000. Further evidence that 3-phosphoinositide-dependent protein kinase-1 (PDK1) is required for the stability and phosphorylation of protein kinase C (PKC) isoforms. *FEBS Lett,* 484**,** 217-23.

BARATA, J. T. 2011. The impact of PTEN regulation by CK2 on PI3K-dependent signaling and leukemia cell survival. *Adv Enzyme Regul,* 51**,** 37-49.

BARBER, D. F., ALVARADO-KRISTENSSON, M., GONZALEZ-GARCIA, A., PULIDO, R. & CARRERA, A. C. 2006. PTEN regulation, a novel function for the p85 subunit of phosphoinositide 3-kinase. *Sci STKE,* 2006**,** pe49.

BARRETT, D., BROWN, V. I., GRUPP, S. A. & TEACHEY, D. T. 2012. Targeting the PI3K/AKT/mTOR signaling axis in children with hematologic malignancies. *Paediatr Drugs,* 14**,** 299-316.

BAYASCAS, J. R. 2010. PDK1: the major transducer of PI 3-kinase actions. *Curr Top Microbiol Immunol,* 346**,** 9-29.

BAYASCAS, J. R., LESLIE, N. R., PARSONS, R., FLEMING, S. & ALESSI, D. R. 2005. Hypomorphic mutation of PDK1 suppresses tumorigenesis in PTEN(+/-) mice. *Curr Biol,* 15**,** 1839-46.

BAYASCAS, J. R., SAKAMOTO, K., ARMIT, L., ARTHUR, J. S. & ALESSI, D. R. 2006. Evaluation of approaches to generation of tissue-specific knock-in mice. *J Biol Chem,* 281**,** 28772-81.

BAYASCAS, J. R., WULLSCHLEGER, S., SAKAMOTO, K., GARCIA-MARTINEZ, J. M., CLACHER, C., KOMANDER, D., VAN AALTEN, D. M., BOINI, K. M., LANG, F., LIPINA, C., LOGIE, L., SUTHERLAND, C., CHUDEK, J. A., VAN DIEPEN, J. A., VOSHOL, P. J., LUCOCQ, J. M. & ALESSI, D. R. 2008. Mutation of the PDK1 PH domain inhibits protein kinase B/Akt, leading to small size and insulin resistance. *Mol Cell Biol,* 28**,** 3258-72.

BILLOTTET, C., GRANDAGE, V. L., GALE, R. E., QUATTROPANI, A., ROMMEL, C., VANHAESEBROECK, B. & KHWAJA, A. 2006. A selective inhibitor of the p110delta isoform of PI 3-kinase inhibits AML cell proliferation and survival and increases the cytotoxic effects of VP16. *Oncogene,* 25**,** 6648-59.

BIONDI, R. M., CHEUNG, P. C., CASAMAYOR, A., DEAK, M., CURRIE, R. A. & ALESSI, D. R. 2000. Identification of a pocket in the PDK1 kinase domain that interacts with PIF and the C-terminal residues of PKA. *EMBO J,* 19**,** 979-88.

BIONDI, R. M., KIELOCH, A., CURRIE, R. A., DEAK, M. & ALESSI, D. R. 2001. The PIF-binding pocket in PDK1 is essential for activation of S6K and SGK, but not PKB. *EMBO J,* 20**,** 4380-90.

BLANK, U., KARLSSON, G. & KARLSSON, S. 2008. Signaling pathways governing stem-cell fate. *Blood,* 111**,** 492-503.

BONE, H. K. & WELHAM, M. J. 2007. Phosphoinositide 3-kinase signalling regulates early development and developmental haemopoiesis. *J Cell Sci,* 120**,** 1752-62.

BONNET, D. & DICK, J. E. 1997. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med,* 3**,** 730-7.

BOROWITZ, M. J., SHUSTER, J., CARROLL, A. J., NASH, M., LOOK, A. T., CAMITTA, B., MAHONEY, D., LAUER, S. J. & PULLEN, D. J. 1997. Prognostic significance of fluorescence intensity of surface marker expression in childhood B-precursor acute lymphoblastic leukemia. A Pediatric Oncology Group Study. *Blood,* 89**,** 3960-6.

BRUNET, A., BONNI, A., ZIGMOND, M. J., LIN, M. Z., JUO, P., HU, L. S., ANDERSON, M. J., ARDEN, K. C., BLENIS, J. & GREENBERG, M. E. 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell,* 96**,** 857-68.

BURGERING, B. M. 2008. A brief introduction to FOXOlogy. *Oncogene,* 27**,** 2258-62.

CAI, J., XU, L., TANG, H., YANG, Q., YI, X., FANG, Y., ZHU, Y. & WANG, Z. 2014. The role of the PTEN/PI3K/Akt pathway on prognosis in epithelial ovarian cancer: a meta-analysis. *Oncologist,* 19**,** 528-35.

CAI, X., GAUDET, J. J., MANGAN, J. K., CHEN, M. J., DE OBALDIA, M. E., OO, Z., ERNST, P. & SPECK, N. A. 2011. Runx1 loss minimally impacts long-term hematopoietic stem cells. *PLoS One,* 6**,** e28430.

CALLEJA, V., LAGUERRE, M., PARKER, P. J. & LARIJANI, B. 2009. Role of a novel PH-kinase domain interface in PKB/Akt regulation: structural mechanism for allosteric inhibition. *PLoS Biol,* 7**,** e17.

CARAVATTA, L., SANCILIO, S., DI GIACOMO, V., RANA, R., CATALDI, A. & DI PIETRO, R. 2008. PI3-K/Akt-dependent activation of cAMP-response element-binding (CREB) protein in Jurkat T leukemia cells treated with TRAIL. *J Cell Physiol,* 214**,** 192-200.

CARNERO, A. & PARAMIO, J. M. 2014. The PTEN/PI3K/AKT Pathway in vivo, Cancer Mouse Models. *Front Oncol,* 4**,** 252.

CARRACEDO, A. & PANDOLFI, P. P. 2008. The PTEN-PI3K pathway: of feedbacks and cross-talks. *Oncogene,* 27**,** 5527-41.

CHALHOUB, N. & BAKER, S. J. 2009. PTEN and the PI3-kinase pathway in cancer. *Annu Rev Pathol,* 4**,** 127-50.

CHALHOUB, N., ZHU, G., ZHU, X. & BAKER, S. J. 2009. Cell type specificity of PI3K signaling in Pdk1- and Pten-deficient brains. *Genes Dev,* 23**,** 1619-24.

CHAPPELL, W. H., STEELMAN, L. S., LONG, J. M., KEMPF, R. C., ABRAMS, S. L., FRANKLIN, R. A., BASECKE, J., STIVALA, F., DONIA, M., FAGONE, P., MALAPONTE, G., MAZZARINO, M. C., NICOLETTI, F., LIBRA, M., MAKSIMOVIC-IVANIC, D., MIJATOVIC, S., MONTALTO, G., CERVELLO, M., LAIDLER, P., MILELLA, M., TAFURI, A., BONATI, A., EVANGELISTI, C., COCCO, L., MARTELLI, A. M. & MCCUBREY, J. A. 2011. Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR inhibitors: rationale and importance to inhibiting these pathways in human health. *Oncotarget,* 2**,** 135-64.

CHATTERJEE, A., CHATTERJEE, U. & GHOSH, M. K. 2013. Activation of protein kinase CK2 attenuates FOXO3a functioning in a PML-dependent manner: implications in human prostate cancer. *Cell Death Dis,* 4**,** e543.

CHEUNG, A. M. & MAK, T. W. 2006. PTEN in the haematopoietic system and its therapeutic indications. *Trends Mol Med,* 12**,** 503-5.

CHIN, Y. R., YUAN, X., BALK, S. P. & TOKER, A. 2014. PTEN-deficient tumors depend on AKT2 for maintenance and survival. *Cancer Discov,* 4**,** 942-55.

CHOWDHURY, S., ONGCHIN, M., WAN, G., SHARRATT, E., BRATTAIN, M. G. & RAJPUT, A. 2013. Restoration of PTEN activity decreases metastases in an orthotopic model of colon cancer. *J Surg Res,* 184**,** 755-60.

CIUFFREDA, L., FALCONE, I., CESTA INCANI, U., DEL CURATOLO, A., CONCIATORI, F., MATTEONI, S., VARI, S., VACCARO, V., COGNETTI, F. &

MILELLA, M. 2014. PTEN expression and function in adult cancer stem cells and prospects for therapeutic targeting. *Adv Biol Regul,* 56**,** 66-80.

COBAS, M., WILSON, A., ERNST, B., MANCINI, S. J., MACDONALD, H. R., KEMLER, R. & RADTKE, F. 2004. Beta-catenin is dispensable for hematopoiesis and lymphopoiesis. *J Exp Med,* 199**,** 221-9.

COLLINS, B. J., DEAK, M., ARTHUR, J. S., ARMIT, L. J. & ALESSI, D. R. 2003. In vivo role of the PIF-binding docking site of PDK1 defined by knock-in mutation. *EMBO J,* 22**,** 4202-11.

CORDES, I., KLUTH, M., ZYGIS, D., RINK, M., CHUN, F., EICHELBERG, C., DAHLEM, R., FISCH, M., HOPPNER, W., WAGNER, W., DOH, O., TERRACCIANO, L., SIMON, R., WILCZAK, W., SAUTER, G. & MINNER, S. 2013. PTEN deletions are related to disease progression and unfavourable prognosis in early bladder cancer. *Histopathology,* 63**,** 670-7.

CORDON-BARRIS, L., PASCUAL-GUIRAL, S., YANG, S., GIMENEZ-LLORT, L., LOPE-PIEDRAFITA, S., NIEMEYER, C., CLARO, E., LIZCANO, J. M. & BAYASCAS, J. R. 2016. Mutation of the 3-phosphoinositide-dependent protein kinase-1 (PDK1) substrate-docking site in the developing brain causes microcephaly with abnormal brain morphogenesis independently of Akt, leading to impaired cognition and disruptive behaviors. *Mol Cell Biol*.

COZZIO, A., PASSEGUE, E., AYTON, P. M., KARSUNKY, H., CLEARY, M. L. & WEISSMAN, I. L. 2003. Similar MLL-associated leukemias arising from self-renewing stem cells and short-lived myeloid progenitors. *Genes Dev,* 17**,** 3029-35.

DANCEY, J. 2010. mTOR signaling and drug development in cancer. *Nat Rev Clin Oncol,* 7**,** 209-19.

DEAN, S. J., PERKS, C. M., HOLLY, J. M., BHOO-PATHY, N., LOOI, L. M., MOHAMMED, N. A., MUN, K. S., TEO, S. H., KOOBOTSE, M. O., YIP, C. H. & RHODES, A. 2014. Loss of PTEN expression is associated with IGFBP2 expression, younger age, and late stage in triple-negative breast cancer. *Am J Clin Pathol,* 141**,** 323-33.

DENNING, G., JEAN-JOSEPH, B., PRINCE, C., DURDEN, D. L. & VOGT, P. K. 2007. A short N-terminal sequence of PTEN controls cytoplasmic localization and is required for suppression of cell growth. *Oncogene,* 26**,** 3930-40.

DICK, J. E. 2008. Stem cell concepts renew cancer research. *Blood,* 112**,** 4793-807.

DOULATOV, S., NOTTA, F., LAURENTI, E. & DICK, J. E. 2012. Hematopoiesis: a human perspective. *Cell Stem Cell,* 10**,** 120-36.

EKLUND, E. 2011. The role of Hox proteins in leukemogenesis: insights into key regulatory events in hematopoiesis. *Crit Rev Oncog,* 16**,** 65-76.

ELKABETS, M., VORA, S., JURIC, D., MORSE, N., MINO-KENUDSON, M., MURANEN, T., TAO, J., CAMPOS, A. B., RODON, J., IBRAHIM, Y. H., SERRA, V., RODRIK-OUTMEZGUINE, V., HAZRA, S., SINGH, S., KIM, P., QUADT, C., LIU, M., HUANG, A., ROSEN, N., ENGELMAN, J. A., SCALTRITI, M. & BASELGA, J. 2013. mTORC1 inhibition is required for sensitivity to PI3K p110alpha inhibitors in PIK3CA-mutant breast cancer. *Sci Transl Med,* 5**,** 196ra99.

ENGELMAN, J. A. 2009. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nat Rev Cancer,* 9**,** 550-62.

FAULK, K., GORE, L. & COOPER, T. 2014. Overview of therapy and strategies for optimizing outcomes in de novo pediatric acute myeloid leukemia. *Paediatr Drugs,* 16**,** 213-27.

FERRO, R. & FALASCA, M. 2014. Emerging role of the KRAS-PDK1 axis in pancreatic cancer. *World J Gastroenterol,* 20**,** 10752-7.

FINLAY, D. K., SINCLAIR, L. V., FEIJOO, C., WAUGH, C. M., HAGENBEEK, T. J., SPITS, H. & CANTRELL, D. A. 2009. Phosphoinositide-dependent kinase 1 controls migration and malignant transformation but not cell growth and proliferation in PTEN-null lymphocytes. *J Exp Med,* 206**,** 2441-54.

FRAGOSO, R. & BARATA, J. T. 2014. PTEN and leukemia stem cells. *Adv Biol Regul,* 56**,** 22-9.

FRANSECKY, L., MOCHMANN, L. H. & BALDUS, C. D. 2015. Outlook on PI3K/AKT/mTOR inhibition in acute leukemia. *Mol Cell Ther,* 3**,** 2.

GAGLIARDI, P. A., DI BLASIO, L., ORSO, F., SEANO, G., SESSA, R., TAVERNA, D., BUSSOLINO, F. & PRIMO, L. 2012. 3-phosphoinositide-dependent kinase 1 controls breast tumor growth in a kinase-dependent but Akt-independent manner. *Neoplasia,* 14**,** 719-31.

GAGLIARDI, P. A., DI BLASIO, L. & PRIMO, L. 2015. PDK1: A signaling hub for cell migration and tumor invasion. *Biochim Biophys Acta,* 1856**,** 178-88.

GAMELL, C., JAN PAUL, P., HAUPT, Y. & HAUPT, S. 2014. PML tumour suppression and beyond: therapeutic implications. *FEBS Lett,* 588**,** 2653-62.

GEORGIADES, P., OGILVY, S., DUVAL, H., LICENCE, D. R., CHARNOCK-JONES, D. S., SMITH, S. K. & PRINT, C. G. 2002. VavCre transgenic mice: a tool for mutagenesis in hematopoietic and endothelial lineages. *Genesis,* 34**,** 251-6.

GLAYSHER, S., BOLTON, L. M., JOHNSON, P., TORRANCE, C. & CREE, I. A. 2014. Activity of EGFR, mTOR and PI3K inhibitors in an isogenic breast cell line model. *BMC Res Notes,* 7**,** 397.

GROVE, C. S. & VASSILIOU, G. S. 2014. Acute myeloid leukaemia: a paradigm for the clonal evolution of cancer? *Dis Model Mech,* 7**,** 941-51.

GUERTIN, D. A. & SABATINI, D. M. 2007. Defining the role of mTOR in cancer. *Cancer Cell,* 12**,** 9-22.

GUERTIN, D. A. & SABATINI, D. M. 2009. The pharmacology of mTOR inhibition. *Sci Signal,* 2**,** pe24.

GUO, W., SCHUBBERT, S., CHEN, J. Y., VALAMEHR, B., MOSESSIAN, S., SHI, H., DANG, N. H., GARCIA, C., THEODORO, M. F., VARELLA-GARCIA, M. & WU, H. 2011. Suppression of leukemia development caused by PTEN loss. *Proc Natl Acad Sci U S A,* 108**,** 1409-14.

GUO, Z., DOSE, M., KOVALOVSKY, D., CHANG, R., O'NEIL, J., LOOK, A. T., VON BOEHMER, H., KHAZAIE, K. & GOUNARI, F. 2007. Beta-catenin stabilization stalls the transition from double-positive to single-positive stage and predisposes thymocytes to malignant transformation. *Blood,* 109**,** 5463-72.

GUPTA, A., YANG, Q., PANDITA, R. K., HUNT, C. R., XIANG, T., MISRI, S., ZENG, S., PAGAN, J., JEFFERY, J., PUC, J., KUMAR, R., FENG, Z., POWELL, S. N., BHAT, A., YAGUCHI, T., WADHWA, R., KAUL, S. C., PARSONS, R.,

KHANNA, K. K. & PANDITA, T. K. 2009. Cell cycle checkpoint defects contribute to genomic instability in PTEN deficient cells independent of DNA DSB repair. *Cell Cycle,* 8**,** 2198-210.

GUTIERREZ, A., SANDA, T., GREBLIUNAITE, R., CARRACEDO, A., SALMENA, L., AHN, Y., DAHLBERG, S., NEUBERG, D., MOREAU, L. A., WINTER, S. S., LARSON, R., ZHANG, J., PROTOPOPOV, A., CHIN, L., PANDOLFI, P. P., SILVERMAN, L. B., HUNGER, S. P., SALLAN, S. E. & LOOK, A. T. 2009. High frequency of PTEN, PI3K, and AKT abnormalities in T-cell acute lymphoblastic leukemia. *Blood,* 114**,** 647-50.

HAFSI, S., PEZZINO, F. M., CANDIDO, S., LIGRESTI, G., SPANDIDOS, D. A., SOUA, Z., MCCUBREY, J. A., TRAVALI, S. & LIBRA, M. 2012. Gene alterations in the PI3K/PTEN/AKT pathway as a mechanism of drug-resistance (review). *Int J Oncol,* 40**,** 639-44.

HALES, E. C., ORR, S. M., LARSON GEDMAN, A., TAUB, J. W. & MATHERLY, L. H. 2013. Notch1 receptor regulates AKT protein activation loop (Thr308) dephosphorylation through modulation of the PP2A phosphatase in phosphatase and tensin homolog (PTEN)-null T-cell acute lymphoblastic leukemia cells. *J Biol Chem,* 288**,** 22836-48.

HAN, S., RITZENTHALER, J. D., ZHENG, Y. & ROMAN, J. 2008. PPARbeta/delta agonist stimulates human lung carcinoma cell growth through inhibition of PTEN expression: the involvement of PI3K and NF-kappaB signals. *Am J Physiol Lung Cell Mol Physiol,* 294**,** L1238-49.

HARRIS, T. E. & LAWRENCE, J. C., JR. 2003. TOR signaling. *Sci STKE,* 2003**,** re15.

HASHIMOTO, K., TSUDA, H., KOIZUMI, F., SHIMIZU, C., YONEMORI, K., ANDO, M., KODAIRA, M., YUNOKAWA, M., FUJIWARA, Y. & TAMURA, K. 2014. Activated PI3K/AKT and MAPK pathways are potential good prognostic markers in node-positive, triple-negative breast cancer. *Ann Oncol,* 25**,** 1973-9.

HE, K., XU, T., XU, Y., RING, A., KAHN, M. & GOLDKORN, A. 2014. Cancer cells acquire a drug resistant, highly tumorigenic, cancer stem-like phenotype through modulation of the PI3K/Akt/beta-catenin/CBP pathway. *Int J Cancer,* 134**,** 43-54.

HOSSEN, M. J., KIM, S. C., YANG, S., KIM, H. G., JEONG, D., YI, Y. S., SUNG, N. Y., LEE, J. O., KIM, J. H. & CHO, J. Y. 2015. PDK1 disruptors and modulators: a patent review. *Expert Opin Ther Pat,* 25**,** 513-37.

HUNG, C. M., GARCIA-HARO, L., SPARKS, C. A. & GUERTIN, D. A. 2012. mTOR-dependent cell survival mechanisms. *Cold Spring Harb Perspect Biol,* 4.

HUNTLY, B. J., SHIGEMATSU, H., DEGUCHI, K., LEE, B. H., MIZUNO, S., DUCLOS, N., ROWAN, R., AMARAL, S., CURLEY, D., WILLIAMS, I. R., AKASHI, K. & GILLILAND, D. G. 2004. MOZ-TIF2, but not BCR-ABL, confers properties of leukemic stem cells to committed murine hematopoietic progenitors. *Cancer Cell,* 6**,** 587-96.

INABA, H., GREAVES, M. & MULLIGHAN, C. G. 2013. Acute lymphoblastic leukaemia. *Lancet,* 381**,** 1943-55.

IWANAMI, A., CLOUGHESY, T. F. & MISCHEL, P. S. 2009. Striking the balance between PTEN and PDK1: it all depends on the cell context. *Genes Dev,* 23**,** 1699-704.

IWASAKI, H. & AKASHI, K. 2007. Hematopoietic developmental pathways: on cellular basis. *Oncogene,* 26**,** 6687-96.

JEANNET, G., SCHELLER, M., SCARPELLINO, L., DUBOUX, S., GARDIOL, N., BACK, J., KUTTLER, F., MALANCHI, I., BIRCHMEIER, W., LEUTZ, A., HUELSKEN, J. & HELD, W. 2008. Long-term, multilineage hematopoiesis occurs in the combined absence of beta-catenin and gamma-catenin. *Blood,* 111**,** 142-9.

KALAITZIDIS, D., SYKES, S. M., WANG, Z., PUNT, N., TANG, Y., RAGU, C., SINHA, A. U., LANE, S. W., SOUZA, A. L., CLISH, C. B., ANASTASIOU, D., GILLILAND, D. G., SCADDEN, D. T., GUERTIN, D. A. & ARMSTRONG, S. A. 2012. mTOR complex 1 plays critical roles in hematopoiesis and Pten-loss-evoked leukemogenesis. *Cell Stem Cell,* 11**,** 429-39.

KARUNANITHI, S., XIONG, T., UHM, M., LETO, D., SUN, J., CHEN, X. W. & SALTIEL, A. R. 2014. A Rab10:RalA G protein cascade regulates insulin-stimulated glucose uptake in adipocytes. *Mol Biol Cell,* 25**,** 3059-69.

KAUL, R., SAHA, P., SARADHI, M., PRASAD, R. L., CHATTERJEE, S., GHOSH, I., TYAGI, R. K. & DATTA, K. 2012. Overexpression of hyaluronan-binding protein 1 (HABP1/p32/gC1qR) in HepG2 cells leads to increased hyaluronan synthesis and cell proliferation by up-regulation of cyclin D1 in AKT-dependent pathway. *J Biol Chem,* 287**,** 19750-64.

KHAN, K. H., YAP, T. A., YAN, L. & CUNNINGHAM, D. 2013. Targeting the PI3K-AKT-mTOR signaling network in cancer. *Chin J Cancer,* 32**,** 253-65.

KIM, R. J., BAE, E., HONG, Y. K., HONG, J. Y., KIM, N. K., AHN, H. J., OH, J. J. & PARK, D. S. 2014. PTEN Loss-Mediated Akt Activation Increases the Properties of Cancer Stem-Like Cell Populations in Prostate Cancer. *Oncology,* 87**,** 270-279.

KINDLER, T., BREITENBUECHER, F., KASPER, S., ESTEY, E., GILES, F., FELDMAN, E., EHNINGER, G., SCHILLER, G., KLIMEK, V., NIMER, S. D., GRATWOHL, A., CHOUDHARY, C. R., MUELLER-TIDOW, C., SERVE, H., GSCHAIDMEIER, H., COHEN, P. S., HUBER, C. & FISCHER, T. 2005. Identification of a novel activating mutation (Y842C) within the activation loop of FLT3 in patients with acute myeloid leukemia (AML). *Blood,* 105**,** 335-40.

KUMANO, K., CHIBA, S., KUNISATO, A., SATA, M., SAITO, T., NAKAGAMI-YAMAGUCHI, E., YAMAGUCHI, T., MASUDA, S., SHIMIZU, K., TAKAHASHI, T., OGAWA, S., HAMADA, Y. & HIRAI, H. 2003. Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells. *Immunity,* 18**,** 699-711.

KUMAR, C. C. 2011. Genetic abnormalities and challenges in the treatment of acute myeloid leukemia. *Genes Cancer,* 2**,** 95-107.

KWAK, Y. G., SONG, C. H., YI, H. K., HWANG, P. H., KIM, J. S., LEE, K. S. & LEE, Y. C. 2003. Involvement of PTEN in airway hyperresponsiveness and inflammation in bronchial asthma. *J Clin Invest,* 111**,** 1083-92.

LAPIDOT, T., SIRARD, C., VORMOOR, J., MURDOCH, B., HOANG, T., CACERES-CORTES, J., MINDEN, M., PATERSON, B., CALIGIURI, M. A. &

DICK, J. E. 1994. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature,* 367**,** 645-8.

LAWLOR, M. A., MORA, A., ASHBY, P. R., WILLIAMS, M. R., MURRAY-TAIT, V., MALONE, L., PRESCOTT, A. R., LUCOCQ, J. M. & ALESSI, D. R. 2002. Essential role of PDK1 in regulating cell size and development in mice. *EMBO J,* 21**,** 3728-38.

LI, N., LEE, B., LIU, R. J., BANASR, M., DWYER, J. M., IWATA, M., LI, X. Y., AGHAJANIAN, G. & DUMAN, R. S. 2010. mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. *Science,* 329**,** 959-64.

LIM, H. J., CROWE, P. & YANG, J. L. 2014. Current clinical regulation of PI3K/PTEN/Akt/mTOR signalling in treatment of human cancer. *J Cancer Res Clin Oncol*.

LOOK, A. T. 1997. Oncogenic transcription factors in the human acute leukemias. *Science,* 278**,** 1059-64.

MAARIFI, G., CHELBI-ALIX, M. K. & NISOLE, S. 2014. PML control of cytokine signaling. *Cytokine Growth Factor Rev*.

MAGEE, J. A., IKENOUE, T., NAKADA, D., LEE, J. Y., GUAN, K. L. & MORRISON, S. J. 2012. Temporal changes in PTEN and mTORC2 regulation of hematopoietic stem cell self-renewal and leukemia suppression. *Cell Stem Cell,* 11**,** 415-28.

MAILLARD, I., WENG, A. P., CARPENTER, A. C., RODRIGUEZ, C. G., SAI, H., XU, L., ALLMAN, D., ASTER, J. C. & PEAR, W. S. 2004. Mastermind critically regulates Notch-mediated lymphoid cell fate decisions. *Blood,* 104**,** 1696-702.

MANNING, B. D. & CANTLEY, L. C. 2007. AKT/PKB signaling: navigating downstream. *Cell,* 129**,** 1261-74.

MASSACESI, C., DI TOMASO, E., URBAN, P., GERMA, C., QUADT, C., TRANDAFIR, L., AIMONE, P., FRETAULT, N., DHARAN, B., TAVORATH, R. & HIRAWAT, S. 2016. PI3K inhibitors as new cancer therapeutics: implications for clinical trial design. *Onco Targets Ther,* 9**,** 203-10.

MAZZA, M. & PELICCI, P. G. 2013. Is PML a Tumor Suppressor? *Front Oncol,* 3**,** 174.

MCCUBREY, J. A., ABRAMS, S. L., UMEZAWA, K., COCCO, L., MARTELLI, A. M., FRANKLIN, R. A., CHAPPELL, W. H. & STEELMAN, L. S. 2012. Novel approaches to target cancer initiating cells-eliminating the root of the cancer. *Adv Biol Regul,* 52**,** 249-64.

MCMANUS, E. J., COLLINS, B. J., ASHBY, P. R., PRESCOTT, A. R., MURRAY-TAIT, V., ARMIT, L. J., ARTHUR, J. S. & ALESSI, D. R. 2004. The in vivo role of PtdIns(3,4,5)P3 binding to PDK1 PH domain defined by knockin mutation. *EMBO J,* 23**,** 2071-82.

MEDYOUF, H., GAO, X., ARMSTRONG, F., GUSSCOTT, S., LIU, Q., GEDMAN, A. L., MATHERLY, L. H., SCHULTZ, K. R., PFLUMIO, F., YOU, M. J. & WENG, A. P. 2010. Acute T-cell leukemias remain dependent on Notch signaling despite PTEN and INK4A/ARF loss. *Blood,* 115**,** 1175-84.

MEINKE, G., BOHM, A., HAUBER, J., PISABARRO, M. T. & BUCHHOLZ, F. 2016. Cre Recombinase and Other Tyrosine Recombinases. *Chem Rev*.

MENDOZA, M. C., ER, E. E. & BLENIS, J. 2011. The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation. *Trends Biochem Sci,* 36**,** 320-8.

MOLOFSKY, A. V., PARDAL, R. & MORRISON, S. J. 2004. Diverse mechanisms regulate stem cell self-renewal. *Curr Opin Cell Biol,* 16**,** 700-7.

MORGAN, K., KHARAS, M., DZIERZAK, E. & GILLILAND, D. G. 2008. Isolation of early hematopoietic stem cells from murine yolk sac and AGM. *J Vis Exp*.

MORRISON, S. J. & KIMBLE, J. 2006. Asymmetric and symmetric stem-cell divisions in development and cancer. *Nature,* 441**,** 1068-74.

MULLIGHAN, C. G., MILLER, C. B., RADTKE, I., PHILLIPS, L. A., DALTON, J., MA, J., WHITE, D., HUGHES, T. P., LE BEAU, M. M., PUI, C. H., RELLING, M. V., SHURTLEFF, S. A. & DOWNING, J. R. 2008. BCR-ABL1 lymphoblastic leukaemia is characterized by the deletion of Ikaros. *Nature,* 453**,** 110-4.

NAJAFOV, A., SHPIRO, N. & ALESSI, D. R. 2012. Akt is efficiently activated by PIF-pocket- and PtdIns(3,4,5)P3-dependent mechanisms leading to resistance to PDK1 inhibitors. *Biochem J,* 448**,** 285-95.

NAKAHARA, F., WEISS, C. N. & ITO, K. 2014. The role of PML in hematopoietic and leukemic stem cell maintenance. *Int J Hematol,* 100**,** 18-26.

OGITA, S. & LORUSSO, P. 2011. Targeting phosphatidylinositol 3 kinase (PI3K)-Akt beyond rapalogs. *Target Oncol,* 6**,** 103-17.

OGURO, H., DING, L. & MORRISON, S. J. 2013. SLAM family markers resolve functionally distinct subpopulations of hematopoietic stem cells and multipotent progenitors. *Cell Stem Cell,* 13**,** 102-16.

PARK, I. K., QIAN, D., KIEL, M., BECKER, M. W., PIHALJA, M., WEISSMAN, I. L., MORRISON, S. J. & CLARKE, M. F. 2003. Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature,* 423**,** 302-5.

PARK, S. G., LONG, M., KANG, J. A., KIM, W. S., LEE, C. R., IM, S. H., STRICKLAND, I., SCHULZE-LUEHRMANN, J., HAYDEN, M. S. & GHOSH, S. 2013. The kinase PDK1 is essential for B-cell receptor mediated survival signaling. *PLoS One,* 8**,** e55378.

PERRY, J. M., HE, X. C., SUGIMURA, R., GRINDLEY, J. C., HAUG, J. S., DING, S. & LI, L. 2011. Cooperation between both Wnt/{beta}-catenin and PTEN/PI3K/Akt signaling promotes primitive hematopoietic stem cell self-renewal and expansion. *Genes Dev,* 25**,** 1928-42.

QUESENBERRY, P. J., GOLDBERG, L., ALIOTTA, J. & DOONER, M. 2014. Marrow Hematopoietic Stem Cells Revisited: They Exist in a Continuum and are Not Defined by Standard Purification Approaches; Then There are the Microvesicles. *Front Oncol,* 4**,** 56.

RAAPHORST, F. M. 2003. Self-renewal of hematopoietic and leukemic stem cells: a central role for the Polycomb-group gene Bmi-1. *Trends Immunol,* 24**,** 522-4.

REYA, T., DUNCAN, A. W., AILLES, L., DOMEN, J., SCHERER, D. C., WILLERT, K., HINTZ, L., NUSSE, R. & WEISSMAN, I. L. 2003. A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature,* 423**,** 409-14.

REYA, T., MORRISON, S. J., CLARKE, M. F. & WEISSMAN, I. L. 2001. Stem cells, cancer, and cancer stem cells. *Nature,* 414**,** 105-11.

ROBERT-MORENO, A., GUIU, J., RUIZ-HERGUIDO, C., LOPEZ, M. E., INGLES-ESTEVE, J., RIERA, L., TIPPING, A., ENVER, T., DZIERZAK, E., GRIDLEY, T., ESPINOSA, L. & BIGAS, A. 2008. Impaired embryonic haematopoiesis yet normal arterial development in the absence of the Notch ligand Jagged1. *EMBO J,* 27**,** 1886-95.

SABATINI, D. M. 2006. mTOR and cancer: insights into a complex relationship. *Nat Rev Cancer,* 6**,** 729-34.

SARBASSOV, D. D., ALI, S. M., SENGUPTA, S., SHEEN, J. H., HSU, P. P., BAGLEY, A. F., MARKHARD, A. L. & SABATINI, D. M. 2006. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol Cell,* 22**,** 159-68.

SARBASSOV, D. D., GUERTIN, D. A., ALI, S. M. & SABATINI, D. M. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science,* 307**,** 1098-101.

SAUER, T., SILLING, G., GROTH, C., ROSENOW, F., KRUG, U., GORLICH, D., EVERS, G., ALBRING, J., BESOKE, R., MESTERS, R. M., MULLER-TIDOW, C., KESSLER, T., BUCHNER, T., BERDEL, W. E. & STELLJES, M. 2015. Treatment strategies in patients with AML or high-risk myelodysplastic syndrome relapsed after Allo-SCT. *Bone Marrow Transplant,* 50**,** 485-92.

SERONT, E., PINTO, A., BOUZIN, C., BERTRAND, L., MACHIELS, J. P. & FERON, O. 2013. PTEN deficiency is associated with reduced sensitivity to mTOR

inhibitor in human bladder cancer through the unhampered feedback loop driving PI3K/Akt activation. *Br J Cancer,* 109**,** 1586-92.

SHEN, G., RONG, X., ZHAO, J., YANG, X., LI, H., JIANG, H., ZHOU, Q., JI, T., HUANG, S., ZHANG, J. & JIA, H. 2014. MicroRNA-105 suppresses cell proliferation and inhibits PI3K/AKT signaling in human hepatocellular carcinoma. *Carcinogenesis*.

SHI, Y., PALUCH, B. E., WANG, X. & JIANG, X. 2012. PTEN at a glance. *J Cell Sci,* 125**,** 4687-92.

SHOJAEE, S., CHAN, L. N., BUCHNER, M., CAZZANIGA, V., COSGUN, K. N., GENG, H., QIU, Y. H., VON MINDEN, M. D., ERNST, T., HOCHHAUS, A., CAZZANIGA, G., MELNICK, A., KORNBLAU, S. M., GRAEBER, T. G., WU, H., JUMAA, H. & MUSCHEN, M. 2016. PTEN opposes negative selection and enables oncogenic transformation of pre-B cells. *Nat Med,* 22**,** 379-87.

SHUCH, B., RICKETTS, C. J., VOCKE, C. D., KOMIYA, T., MIDDELTON, L. A., KAUFFMAN, E. C., MERINO, M. J., METWALLI, A. R., DENNIS, P. & LINEHAN, W. M. 2013. Germline PTEN mutation Cowden syndrome: an underappreciated form of hereditary kidney cancer. *J Urol,* 190**,** 1990-8.

SO, C. W., KARSUNKY, H., PASSEGUE, E., COZZIO, A., WEISSMAN, I. L. & CLEARY, M. L. 2003. MLL-GAS7 transforms multipotent hematopoietic progenitors and induces mixed lineage leukemias in mice. *Cancer Cell,* 3**,** 161-71.

STAAL, F. J. & LUIS, T. C. 2010. Wnt signaling in hematopoiesis: crucial factors for self-renewal, proliferation, and cell fate decisions. *J Cell Biochem,* 109**,** 844-9.

STEELMAN, L. S., BERTRAND, F. E. & MCCUBREY, J. A. 2004. The complexity of PTEN: mutation, marker and potential target for therapeutic intervention. *Expert Opin Ther Targets,* 8**,** 537-50.

STEELMAN, L. S., NAVOLANIC, P. M., SOKOLOSKY, M. L., TAYLOR, J. R., LEHMANN, B. D., CHAPPELL, W. H., ABRAMS, S. L., WONG, E. W., STADELMAN, K. M., TERRIAN, D. M., LESLIE, N. R., MARTELLI, A. M., STIVALA, F., LIBRA, M., FRANKLIN, R. A. & MCCUBREY, J. A. 2008. Suppression of PTEN function increases breast cancer chemotherapeutic drug resistance while conferring sensitivity to mTOR inhibitors. *Oncogene,* 27**,** 4086-95.

STIER, S., CHENG, T., DOMBKOWSKI, D., CARLESSO, N. & SCADDEN, D. T. 2002. Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome. *Blood,* 99**,** 2369-78.

SUJOBERT, P., BARDET, V., CORNILLET-LEFEBVRE, P., HAYFLICK, J. S., PRIE, N., VERDIER, F., VANHAESEBROECK, B., MULLER, O., PESCE, F., IFRAH, N., HUNAULT-BERGER, M., BERTHOU, C., VILLEMAGNE, B., JOURDAN, E., AUDHUY, B., SOLARY, E., WITZ, B., HAROUSSEAU, J. L., HIMBERLIN, C., LAMY, T., LIOURE, B., CAHN, J. Y., DREYFUS, F., MAYEUX, P., LACOMBE, C. & BOUSCARY, D. 2005. Essential role for the p110delta isoform in phosphoinositide 3-kinase activation and cell proliferation in acute myeloid leukemia. *Blood,* 106**,** 1063-6.

SZCZEPANSKI, T., VAN DER VELDEN, V. H. & VAN DONGEN, J. J. 2003. Classification systems for acute and chronic leukaemias. *Best Pract Res Clin Haematol,* 16**,** 561-82.

TAN, J., LI, Z., LEE, P. L., GUAN, P., AAU, M. Y., LEE, S. T., FENG, M., LIM, C. Z., LEE, E. Y., WEE, Z. N., LIM, Y. C., KARUTURI, R. K. & YU, Q. 2013. PDK1 signaling toward PLK1-MYC activation confers oncogenic transformation, tumor-initiating cell activation, and resistance to mTOR-targeted therapy. *Cancer Discov,* 3**,** 1156-71.

TANG, P., GAO, C., LI, A., ASTER, J., SUN, L. & CHAI, L. 2013. Differential roles of Kras and Pten in murine leukemogenesis. *Leukemia,* 27**,** 1210-4.

TESIO, M., OSER, G. M., BACCELLI, I., BLANCO-BOSE, W., WU, H., GOTHERT, J. R., KOGAN, S. C. & TRUMPP, A. 2013. Pten loss in the bone marrow leads to G-CSF-mediated HSC mobilization. *J Exp Med,* 210**,** 2337-49.

TROTMAN, L. C., WANG, X., ALIMONTI, A., CHEN, Z., TERUYA-FELDSTEIN, J., YANG, H., PAVLETICH, N. P., CARVER, B. S., CORDON-CARDO, C., ERDJUMENT-BROMAGE, H., TEMPST, P., CHI, S. G., KIM, H. J., MISTELI, T., JIANG, X. & PANDOLFI, P. P. 2007. Ubiquitination regulates PTEN nuclear import and tumor suppression. *Cell,* 128**,** 141-56.

VANHAESEBROECK, B., STEPHENS, L. & HAWKINS, P. 2012. PI3K signalling: the path to discovery and understanding. *Nat Rev Mol Cell Biol,* 13**,** 195-203.

VENIGALLA, R. K., MCGUIRE, V. A., CLARKE, R., PATTERSON-KANE, J. C., NAJAFOV, A., TOTH, R., MCCARTHY, P. C., SIMEONS, F., STOJANOVSKI, L. & ARTHUR, J. S. 2013. PDK1 regulates VDJ recombination, cell-cycle exit and survival during B-cell development. *EMBO J,* 32**,** 1008-22.

VOGT, P. K., BADER, A. G. & KANG, S. 2006. PI 3-kinases: hidden potentials revealed. *Cell Cycle,* 5**,** 946-9.

WANG, Z., ZHONG, J., INUZUKA, H., GAO, D., SHAIK, S., SARKAR, F. H. & WEI, W. 2012. An evolving role for DEPTOR in tumor development and progression. *Neoplasia,* 14**,** 368-75.

WARR, M. R., PIETRAS, E. M. & PASSEGUE, E. 2011. Mechanisms controlling hematopoietic stem cell functions during normal hematopoiesis and hematological malignancies. *Wiley Interdiscip Rev Syst Biol Med,* 3**,** 681-701.

WETZKER, R. & ROMMEL, C. 2004. Phosphoinositide 3-kinases as targets for therapeutic intervention. *Curr Pharm Des,* 10**,** 1915-22.

WYMANN, M. P., BULGARELLI-LEVA, G., ZVELEBIL, M. J., PIROLA, L., VANHAESEBROECK, B., WATERFIELD, M. D. & PANAYOTOU, G. 1996. Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. *Mol Cell Biol,* 16**,** 1722-33.

XU, J., ZHOU, J. Y., WEI, W. Z. & WU, G. S. 2010. Activation of the Akt survival pathway contributes to TRAIL resistance in cancer cells. *PLoS One,* 5**,** e10226.

XU, Q., SIMPSON, S. E., SCIALLA, T. J., BAGG, A. & CARROLL, M. 2003. Survival of acute myeloid leukemia cells requires PI3 kinase activation. *Blood,* 102**,** 972-80.

YANG, L., BRYDER, D., ADOLFSSON, J., NYGREN, J., MANSSON, R., SIGVARDSSON, M. & JACOBSEN, S. E. 2005. Identification of Lin(-)Sca1(+)kit(+)CD34(+)FLT3- short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients. *Blood,* 105**,** 2717-23.

YEUNG, J. & SO, C. W. 2009. Identification and characterization of hematopoietic stem and progenitor cell populations in mouse bone marrow by flow cytometry. *Methods Mol Biol,* 538**,** 301-15.

YILMAZ, O. H., KIEL, M. J. & MORRISON, S. J. 2006a. SLAM family markers are conserved among hematopoietic stem cells from old and reconstituted mice and markedly increase their purity. *Blood,* 107**,** 924-30.

YILMAZ, O. H., VALDEZ, R., THEISEN, B. K., GUO, W., FERGUSON, D. O., WU, H. & MORRISON, S. J. 2006b. Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. *Nature,* 441**,** 475-82.

ZEISIG, B. B., KULASEKARARAJ, A. G., MUFTI, G. J. & SO, C. W. 2012. SnapShot: Acute myeloid leukemia. *Cancer Cell,* 22**,** 698-698 e1.

ZHANG, H., BAJRASZEWSKI, N., WU, E., WANG, H., MOSEMAN, A. P., DABORA, S. L., GRIFFIN, J. D. & KWIATKOWSKI, D. J. 2007. PDGFRs are critical for PI3K/Akt activation and negatively regulated by mTOR. *J Clin Invest,* 117**,** 730-8.

ZHANG, J., GRINDLEY, J. C., YIN, T., JAYASINGHE, S., HE, X. C., ROSS, J. T., HAUG, J. S., RUPP, D., PORTER-WESTPFAHL, K. S., WIEDEMANN, L. M., WU, H. & LI, L. 2006. PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. *Nature,* 441**,** 518-22.

ZHANG, J., ROBERTS, T. M. & SHIVDASANI, R. A. 2011. Targeting PI3K signaling as a therapeutic approach for colorectal cancer. *Gastroenterology,* 141**,** 50-61.

ZHANG, P. Y., ZHANG, W. G., HE, A. L., WANG, J. L. & LI, W. B. 2009. Identification and functional characterization of the novel acute monocytic leukemia associated antigen MLAA-34. *Cancer Immunol Immunother,* 58**,** 281-90.

ZHOU, J., DU, T., LI, B., RONG, Y., VERKHRATSKY, A. & PENG, L. 2015. Crosstalk Between MAPK/ERK and PI3K/AKT Signal Pathways During Brain Ischemia/Reperfusion. *ASN Neuro,* 7.